PHYTOESTROGENIC EXTRACTS OF *CYCLOPIA* MODULATE MOLECULAR TARGETS INVOLVED IN THE PREVENTION AND TREATMENT OF BREAST CANCER.

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Dissertation presented for the degree PhD in Biochemistry in the Faculty of Natural Sciences at Stellenbosch University.

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April 2014

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Summary

Phytoestrogen containing extracts of *Cyclopia*, an indigenous South African fynbos plant used to prepare honeybush tea, may serve as a source of new estrogen analogues. It would be of great benefit if these new analogues would not only prevent the development and progression of breast cancer which, globally, is responsible for the highest number of cancer associated deaths among females, but also have a reduced side-effect profile when compared to current treatments and, in addition, also alleviate menopause associated symptoms. In this study three extracts, P104, SM6Met, and cup-of-tea, from two species of *Cyclopia*, *C. genistoides* and *C. subternata*, were evaluated for their potential to modulate molecular targets involved in prevention and treatment of breast cancer. We show that the phytoestrogenic extracts of *Cyclopia* antagonise estrogen-induced cell proliferation both *in vitro* as well as *in vivo*. Furthermore, our study presents various molecular mechanisms whereby the *Cyclopia* extracts may be eliciting this effect. Importantly, we show, for the first time, that the *Cyclopia* extracts behave as ERα antagonists and ERβ agonists which, with respect to the known role of the ER subtypes in breast cancer, where the ERα subtype is associated with the stimulation of cell proliferation and the occurrence of breast cancer, while ERβ ameliorates the action of ERα in breast cancer and could act as an inhibitor of breast cancer development, may be beneficial for the prevention or treatment of breast cancer. In addition, we also show that the extracts of *Cyclopia* behave as selective estrogen receptor degraders by down-regulating ERα protein levels while stabilising ERβ protein levels, which not only provides a possible molecular explanation for the observed ERα antagonism and ERβ agonism, but, in addition, may be beneficial as higher ERα levels are associated with malignant breast cancer tumours, while higher ERβ levels are associated with benign tumours. Furthermore, we show that the *Cyclopia* extracts affect the nuclear localization and distribution of both ER subtypes in a manner that provides an additional molecular explanation for the observed ERα antagonism and ERβ agonism. Investigation of the molecular processes involved in the promotion and progression of breast cancer, such as the

distribution of cells between the phases of the cell cycle, cancer cell invasion, and the regulation of genes governing these processes provides evidence that the *Cyclopia* extracts are not as proliferative as estrogen. In addition, *Cyclopia* extracts display anti-inflammatory properties, which may be beneficial as inflammation is an enabling characteristic in cancer development and progression. Furthermore, this study, for the first time, shows that the phytoestrogenic extracts of *Cyclopia* are absorbed, are not toxic, and display biological ERα antagonist activity *in vivo* by retarding uterine growth. Thus, we propose that the *Cyclopia* extracts act as selective estrogen receptor subtype modulators with potential to be developed as a nutraceutical for the treatment or prevention of breast cancer.

Opsomming

Fitoëstrogeen-bevattende ekstrakte van *Cyclopia*, ‘n inheemse Suid Afrikaanse fynbosplant wat gebruik word vir die voorbereiding van heuningbostee, mag as ‘n bron van nuwe estrogeen-analoë dien. Dit sal baie voordelig wees indien hierdie nuwe analoë nie net die ontwikkeling en progressie van borskanker sal voorkom nie, aangesien borskanker wêreldwyd verantwoordelik is vir die grootste getal kankerverwante sterftes onder vroue, maar ook ‘n verminderde newe-effek profiel vertoon in vergelyking met huidige behandelings en ook, boonop, simptome wat met menopouse geassosieer word, sal verlig. In hierdie studie is drie ekstrakte, P104, SM6Met, en cup-of-tea, vanaf twee spesies van *Cyclopia*, *C. genistoides* en *C. subternata*, geëvalueer vir hul potensiaal om die molekulêre teikens betrokke by die voorkoming en behandeling van borskanker te moduleer. Ons wys dat die fitoëstrogeniese ekstrakte van *Cyclopia* antagoniseer estrogeen-geïnduseerde selproliferasie beide *in vitro* as ook *in vivo*. Verder bied ons studie ook verkskeie molekulêre meganismes aan oor hoe die *Cyclopia* ekstrakte hierdie effek mag ontlok. ‘n Belangrike bevinding is dat ons vir die eerste keer wys dat die *Cyclopia* ekstrakte hulself as ERα -antagoniste en ERβ-agoniste gedra wat, met betrekking tot die erkende rol van die ER-subtipes in borskanker, waar die ERα-subtipe geassosieer word met die stimulasie van selproliferasie en die gebeurtenis van borskanker, terwyl ERβ die aksie van ERα onderdruk en as ‘n inhibeerder van borskankerontwikkeling kan dien, voordelig mag wees vir die voorkoming of behandeling van borskanker. Ons wys boonop ook dat die ekstrakte van *Cyclopia* hulself soos selektiewe estrogeen- reseptor-degradeerders gedra deurdat hul ERα-proteïnvlakke verlaag terwyl hul ERβ-proteïnvlakke stabiliseer. Dit verksaf nie net ‘n moontlike molekulêre verduideliking vir die waargeneemde ERα-antagonisme en ERβ-agonisme nie, maar mag ook voordelig wees in borskanker aangesien hoër ERα-vlakke geasosieer word met kwaadaardige borskankertumors en hoër ERβ-vlakke met nie-kwaadaardige tumors. Verder wys ons dat die *Cyclopia* ekstrakte die lokalisering en verspreiding van beide ER-subtipes in die selkern op so ‘n wyse beïnvloed dat dit ‘n addisionele

molekulêre verduideliking bied vir die ERα-antagonisme en ERβ-agonisme wat waargeneem is. Verdere ondersoek van die molekulêre prosesse betrokke by die promosie en progressie van borskanker, soos die verspreiding van selle tussen die fases van die selsiklus, die beweging van kankerselle na omliggende weefsels, en die regulering van gene wat hierdie prosesse beheer, verskaf bewyse dat die *Cyclopia*-ekstrakte nie so proliferatief is soos estrogeen nie. Die ekstrakte van *Cyclopia* vertoon boonop ook anti-inflamatoriese eienskappe, wat voordelig mag wees aangesien inflammasie ‘n bydraende eienskap in kankerontwikkeling en -progressie is. Verder wys hierdie studie vir die eerste keer dat die fitoëstrogeniese ekstrakte van *Cyclopia* geabsorbeer word, nie toksies is nie, en dat hulle biologiese ERα-antagonis aktiwiteit vertoon deurdat hulle uterus-groei vertraag *in vivo*. Dus stel ons voor dat die *Cyclopia*-ekstrakte optree soos selektiewe-estrogeen-reseptor-subtipe-moduleerders met die potensiaal om ontwikkel te word as ‘n nutraseutiese middel vir die behandeling of voorkoming van borskanker.

I would like to dedicate this dissertation to Stephan Meyer Jnr. and Michelle Visser. Thank you for all your support and encouragement and for always believing in me.

Acknowledgements

Prof. Ann Louw, my supervisor, thank you for believing in me and for allowing me the opportunity to pursue this study. Your patience, guidance and understanding in difficult times were invaluable and are greatly appreciated.

To the members of the Louw and Africander research groups, thank you for all your support and encouraging words.

Carmen Langeveldt, thank you for your impeccable tissue culture work, your support and encouragement.

The South African National Research Foundation (NRF), thank you for financial support.

Thank you to all my family and friends for your support.

Alphabetical list of abbreviations

3βHSD2	3β-hydroxysteroid dehydrogenase/isomerase type 2
17βHSDs	17β-hydroxysteroid dehydrogenases/isomerases
ACTB	Beta-actin
AF	Transcriptional activation function
AFB_1	Aflatoxin B1
AI	Aromatase inhibitor
Akt	Protein kinase B
AP-1	Activating protein-1
ATM	Araxia telangiectasia mutated
B2M	Beta-2 microglobulin
BC	Breast cancer
Bcl-2	B-cell lymphoma 2
BPA	Bisphenol A
BRCA	Breast cancer gene
BRET	Bioluminescent resonance energy transfer
BW	Body weight
CDK	Cyclin-dependent kinase
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CE	Catechol estrogens
C/EBPβ	CCAAT/enhancer-binding protein beta
CE-Q	Catechol estrogen quinones
CPM	Counts per minute

CV	Coefficient of variation
CYP11A1	Cytochrome P450, family 11, subfamily A, polypeptide 1
CYP17A1	Cytochrome P450, family 17, subfamily A, polypeptide 1
CYP19A1	Cytochrome P450, family 19, subfamily A, polypeptide 1
DBD	DNA binding domain
DHEA	Dehydroepiandrosterone
DME	Dried methanol extract
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DPM	Disintegrations per minute
DPN	Diarylpropionitrile
E_1	Estrone
E_2	17β-Estradiol/Estrogen
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
ER	Estrogen receptor
ERE	Estrogen response element
ET	Endocrine therapy
FB_1	Fumonisin B1

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GnRH	Gonadotropin hormone-releasing hormone
GR	Glucocorticoid receptor
GSH	Glutathione
HIF-1	Hypoxia-inducible factor 1
HPLC	High-performance liquid chromatography
HPO axis	Hypothalamic-pituitary-ovarian axis
HPRT1	Hypoxanthine-guanine phosphoribosyltransferase
HRP	Horseradish peroxidase
HRT	Hormone replacement therapy
HSP	Heat shock protein
IGF-1	Insulin-like growth factor-1
IL-6	Interleukin-6
LBD	Ligand binding domain
LHRH	Luteinizing hormone- releasing hormone
MAPK	Mitogen-activated protein kinase
MMP-1	Matrix metalloproteinase-1
MPP	Methyl-piperidino-pyrazole
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NFκB	Nuclear factor-kappa B
NO	Nitric oxide
p53	Tumour protein 53

PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Proteasome inhibitor I/Propidium iodide
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
RNA	Ribonucleic acid
ROI	Region of interest
ROS	Reactive oxygen species
RPL13A	60S ribosomal protein L13a
SDS	Sodium dodecyl sulphate
SERD	Selective estrogen receptor degrader
SERM	Selective estrogen receptor modulator
SERSM	Selective estrogen receptor subtype modulator
SHBG	Sex hormone binding globulin
TF	Transcription factor
THC	*R, R* enantiomer of 5,11-*cis*-diethyl-5,6,11,12-tetrahydrochrysene-2,8-diol
WCB	Whole-cell binding
WHO	World health organization
YFP	Yellow fluorescent protein

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Chapter 1

Introduction to the dissertation

Globally, a trend towards the use of traditional or alternative medicines is emerging: in Japan more than 50% of allopathic doctors prescribe traditional medicines, in the United Kingdom 7-48% of cancer patients have reported using botanical drugs after diagnosis, and in the United States the number of visits to alternative medicine providers exceed the number of visits to primary care physicians [1-3]. Traditional and alternative medicines encompass a variety of treatments, including traditional Chinese medicines, indigenous medicines, as well as herbal medicines [4]. According to the World Health Organization (WHO), herbal medicines include herbs, herbal materials, herbal preparations and finished herbal products, that contain as active ingredients parts of plants, or other plant materials, or combinations thereof [4].

Traditionally, herbal medicines are prepared by either steeping or heating of the unprocessed plant material to produce an herbal tea [5]. The indigenous plant biome of South Africa has delivered various plants that are traditionally consumed as herbal teas, such as rooibos tea (*Aspalathus linearis*), honeybush tea (*Cyclopia* species), and bush tea (*Athrixia phylicoides*) [6]. However, only rooibos and honeybush tea, due to their sweet taste and aroma, have successfully moved into the commercial market [6]. With regards to the use of the South African herbal teas as alternative medicines, extracts of *A. linearis*, which include hot water extracts, as well as constituents of these extracts, have been shown to potentially have anti-obesity [7], anti-diabetic [8-11], anti-cancer [12-14], as well as anti-oxidant [15,16] properties. In addition, extracts of *A. linearis* has been shown to maintain normal glucocorticoid levels and may have applications in the management of stress related conditions and metabolic diseases [17]. Extracts and constituents of *A. phylicoides* have been shown to have anti-oxidant properties [18,19], which is comparable to that of *A. linearis* [18], and could potentially ameliorate metabolic disorders related to obesity and diabetes [20]. Therefore, it becomes clear that herbal teas prepared from indigenous South African plants may have health promoting properties and have potential to be further developed as alternative medicines or nutraceuticals. However, the focus of the current study will be on phytoestrogenic

extracts of *Cyclopia* and their potential use as an alternative treatment for estrogen related disorders such as menopause associated side-effects as well as breast cancer development and progression.

The current study expands on anecdotal findings that associates the consumption of honeybush tea with the stimulation of milk production in breast feeding women [21] and the alleviation of menopausal symptoms [22], as well as scientific findings that have shown that the plant material of several *Cyclopia* species contain various polyphenols with known phytoestrogenic properties [23]. In addition, it has been shown that extracts of *Cyclopia* can bind to both subtypes of the estrogen receptor (ER) [22,24] and although they induce the proliferation of breast cancer cells, they do so with both a lower potency and efficacy than that of estrogen [25] and, in addition, antagonize estrogen induced breast cancer cell proliferation [24].

The remainder of this dissertation consists of five chapters: Chapter 2 provides a literature overview, addressing the development and progression of cancer with the focus on breast cancer and the role of estrogen, the ER subtypes, and the signaling pathways under their control in breast cancer development and progression. In addition, it provides an overview of current therapeutic strategies for the treatment or prevention of breast cancer. Furthermore, the literature review includes a general overview of phytoestrogens and their potential application in the treatment or prevention of breast cancer. Lastly, it includes a section about *Cyclopia* and highlights findings of a review article (Addendum A [23]), of which I am a contributing author, which are relevant to the current study. The literature review aims to validate our decision of identifying the ER subtypes and the signaling pathways under their control as molecular targets for the treatment or prevention of breast cancer and to enlighten the reader about the potential of phytoestrogens and *Cyclopia* extracts specifically, for the development of a nutraceutical for the treatment or prevention of breast cancer. Chapters 3 to 5, of which chapter 3 has been published in the Public Library of Science ONE (PloS One) (PLoS ONE 2013; 8(11): e79223. doi:10.1371/journal.pone.0079223), will each address one of our main aims and Chapter 6 is a final discussion about our combined findings. In chapters 3 to 5 we address the following aims:

- Investigate the possibility of ERα antagonism as well as ERβ agonism by three *Cyclopia* extracts, dried methanol extracts (DMEs) of both *C. subternata* and *C. genistoides*, as well as a water extract of *C. subternata* using both an *in vitro* transactivation and transrepression model where the ER subtypes are either co-expressed or individually. In addition, we also investigate ERα antagonism, *in vivo*, by using the immature rat uterotrophic assay (Chapter 3).
- Investigate the effect of the three *Cyclopia* extracts on the ER subtype protein levels as well as how treatment with these extracts modulate the ERα:ERβ ratio. We will also evaluate how the *Cyclopia* extracts affect the nuclear localization and distribution of the ER subtypes (Chapter 4).
- Investigate how the *Cyclopia* extracts affect aspects of breast cancer promotion and progression in a breast cancer cell line, by evaluating how the *Cyclopia* extracts affect the distribution of cells between the phases of the cell cycle, the invasive potential of breast cancer cells, and the regulation of genes involved in breast cancer cancer promotion, progression, and survival (Chapter 5).

In addition, for each of our aims, we will investigate an example from each of the major classes of phytoestrogens: genistein, a well-studied isoflavone, enterodiol, a lignin, and coumestrol, a coumestan [26,27]. Luteolin, an estrogenic polyphenol [23], will also be included as it was found to be present in all of the *Cyclopia* extracts, while E_2 represents the major endogenous estrogen [28,29]. By including these compounds we would be able to compare the effect of the *Cyclopia* extracts to that of the known phytoestrogens, as well as E_2, and, in addition, where possible, use literature findings regarding these compounds to try and elucidate the mechanism of action of the *Cyclopia* extracts.

Furthermore, the results chapters, chapters 3 to 5, are written in manuscript format and each one contains an introduction, materials and methods, results, and discussion section and therefore some repetition between chapters will inevitably occur.

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Chapter 2

Literature review

Menopause, which occurs naturally during midlife (42-58 years of age), signals the end of the fertile or reproductive phase of a woman's life and is characterised by a decrease of up to 90% in the natural production of estrogen [1]. Menopause can be either a naturally occurring process linked to ageing [1-3] or can be brought on by the removal of the uterus and ovaries during a hysterectomy (surgical menopause) [4]. The onset of menopause, with the characterized decrease in estrogen production, is accompanied by a plethora of side effects that may affect the quality of life of an individual. These side effects include, but are not limited to, vasomotor symptoms such as hot flushes, night sweats, sleeping problems, and vaginal dryness [2,4]. In addition, decreased estrogen production is also a major risk factor for osteoporosis [5].

Hormone replacement therapy (HRT) is traditionally prescribed to women undergoing menopausal transition to alleviate symptoms associated with menopause. Women without a uterus (hysterectomy) are prescribed estrogen alone, while women with an intact uterus are prescribed estrogen in combination with progestins [6]. Although being very effective in alleviating menopausal symptoms, a number of side effects have been associated with the use of HRT, for example, an increased occurrence of vaginal bleeding [7], heart disease or strokes [8,9], and breast cancer [8,10].

Cancer is a disease that is the leading cause of mortality in economically developed countries and the second leading cause of death in developing countries [11]. Amongst women, breast cancer is the most frequently diagnosed cancer (23% globally and 29% in the United States) and is responsible for the highest number of cancer associated deaths (14% globally, 14% in the United States, and 15% in the European Union) [11-13]. Furthermore, breast cancer occurs most frequently in post-menopausal women, which is also the group who would be interested in using HRT [14,15]. Therefore, although globally there is a need to better understand the cause of cancer development and progression to prevent cancer development and/or develop efficient treatments once diagnosed if, in addition, these treatments could also alleviate symptoms associated with the menopausal transition they may be considered prophylactic regarding breast cancer development.

For the purpose of this literature review, I will discuss the molecular mechanism(s) of cancer development and progression by focussing on breast cancer and elaborate on how knowledge of these mechanism(s) are currently, or in future may be, implemented for the development of treatments that may either prevent the development of breast cancer or inhibit, and possibly reverse, the progression thereof. Furthermore, I will introduce the reader to *Cyclopia*, as the modulation of molecular targets involved in the prevention and treatment of breast cancer by the phytoestrogenic extracts of *Cyclopia* is the topic of my dissertation.

2.1. The development and progression of cancer with the focus on breast cancer.

Exogenous estrogen in the form of HRT contributes to cumulative and excessive exposure to estrogens [16,17], which may be considered as one of the most important risk factors for developing breast cancer as it not only increases the exposure to carcinogenic estrogen metabolites [16-23], but, as increased estrogen driven proliferation reduces the amount of time allocated for DNA maintenance the resulting increased mutation frequency may contribute to the development of breast cancer [24,25]. Furthermore, although the incidence of breast cancer is higher in women using HRT [10,26,27], the occurrence thereof is not limited to this group. Therefore, other risk factors that contribute towards excessive and cumulative estrogen exposure have to be considered. These factors include, but is not limited to, early menarche age [28-30], late onset of menopause [30-32], first full term pregnancy at a late age [30,31], and obesity [33,34].

Under normal physiological conditions the human cell will grow, divide and undergo apoptosis to sustain tissue structure and function. Furthermore, this cycle of growth, division, and apoptosis is under strict regulation, which is exerted by the production and release of growth promoting, as well as, pro-and anti-apoptotic signals, and disruptions of this tight regulation can result in abnormal cell growth [35-37], that is the basis of cancer development and progression [38]. Cancer development is a long-term process and for several types of human cancer, including breast cancer, three steps of cancer formation may be distinguished: (1) initiation (genomic DNA damage), (2) promotion (initiated cells grow and divide to form an actively proliferating multi-cellular premalignant tumour

cell population), and (3) progression (production of tumour cells with increased proliferative capacity, invasiveness, and metastatic potential) (Fig. 1) [39-42].

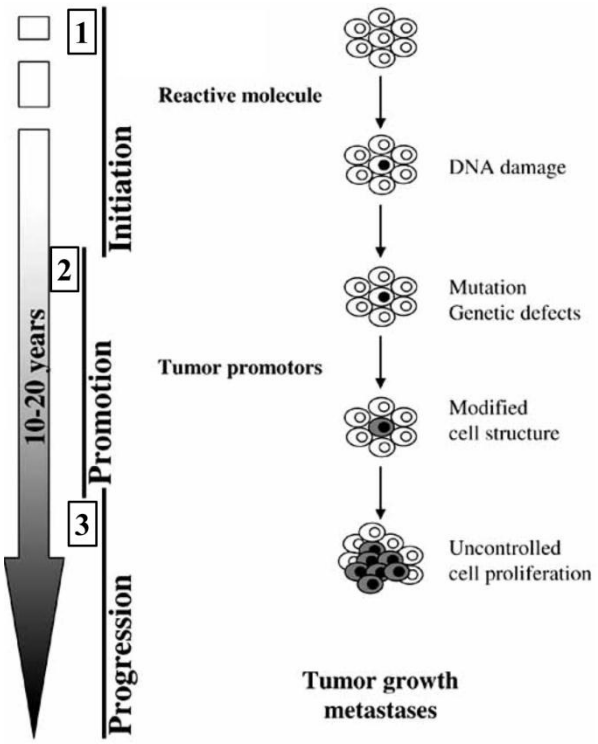

Figure 1. The development and progression of cancer. Cancer development is a long-term process and for several types of human cancer there are three steps of cancer formation: (1) initiation, (2) promotion, and (3) progression. Taken and adapted from Fimognari *et al.* [42]

2.1.1. Initiation

Initiation (Fig. 1), an irreversible process, occurs when the genomic DNA of a normal cell undergoes damage that is either not repaired or repaired incorrectly [42,43]. The main cause of DNA damage is oxidative damage inflicted by reactive oxygen species (ROS) [43-45], which may be produced by exogenous (radiation, environmental agents, pharmaceuticals, or industrial chemicals) or endogenous (mitochondria, peroxisomes, or activation of inflammatory cells) sources [45-47]. If DNA damage occurs, followed by inadequate repair of the damage, the mutated damaged cell may undergo mitosis to generate various clones of the mutated cell [42]. With regards to breast cancer, specific metabolites of estrogens (estrone, E_1, and estradiol, E_2), sex hormones traditionally linked to the development of the female reproductive tract and secondary sex characteristics [48-50], may behave as endogenous chemical carcinogens [16-23]. The major

metabolites of estrogens are catechol estrogens (CE) which, in the event of excessive CE formation, are oxidised to form catechol estrogen quinones (CE-Q) [18]. If insufficient inactivation of CE-Qs by glutathione (GSH) occurs, CE-Qs react with DNA to form stable and depurinating adducts (DNA covalently bonded to a carcinogen with release of a nucleic base), which results in apurinic sites in the DNA (Fig. 2) [18,20-22,51]. Inadequate repair of the apurinic sites in DNA is believed to generate mutations that may initiate estrogen induced cancers such as breast cancer [18,20-22]. In addition, endogenous or exogenous estrogens increase cell division and consequently cell proliferation and this increased cell proliferation rate reduces the amount of time allocated to DNA maintenance resulting in an increased mutation frequency [24,25].

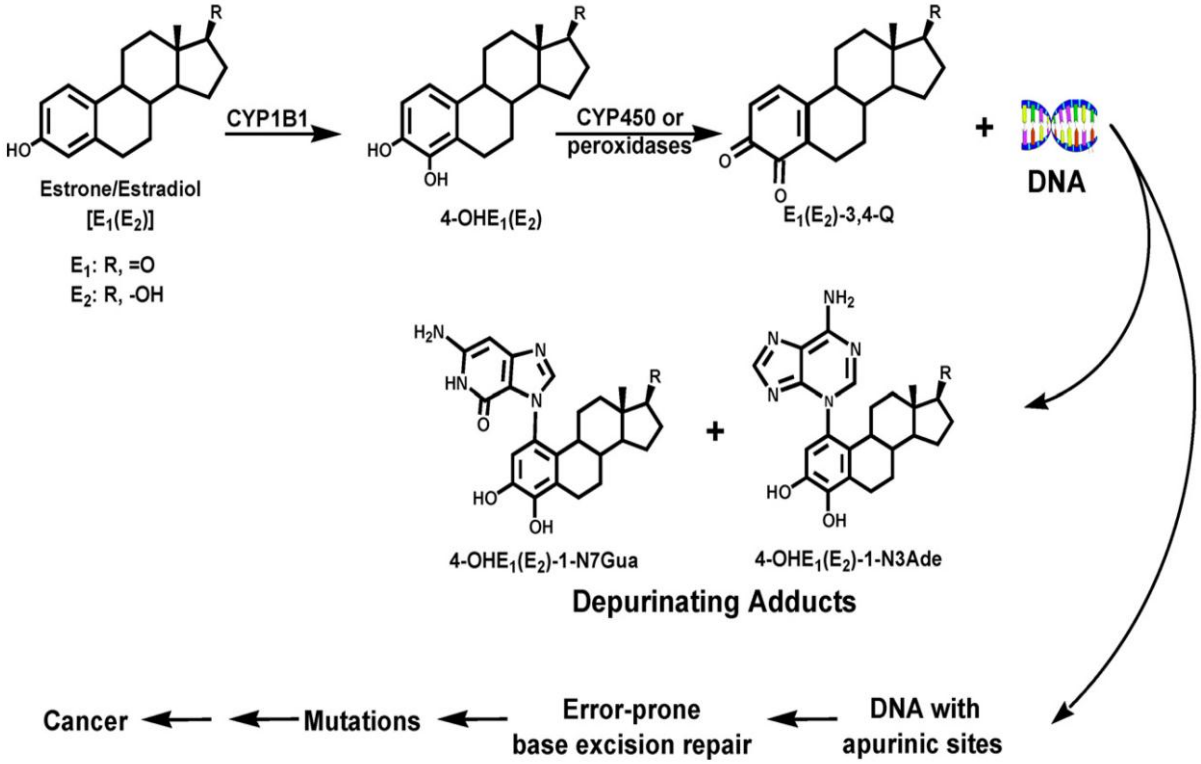

Figure 2. Major metabolic pathway in cancer initiation by estrogens. Cathecol estrogen quinones react with DNA to form depurinating adducts which result in apurininc sites in the DNA. CYP1B1, Cytochrome P450, family 1, subfamily B, polypeptide 1 ; CYP450, Cytochrome P450; DNA, Deoxyribonucleic acid. Figure taken from Cavalieri *et al.* [21].

Furthermore, defects in DNA maintenance, which includes detecting damage, repairing damaged DNA, and inactivating carcinogenic molecules before DNA damage occurs, may increase susceptibility to cancer initiation [35,52-54]. Defective DNA maintenance may be due to hereditary mutations in genes whose products are involved in DNA maintenance, thereby increasing an

individual's vulnerability to develop hereditary cancers [54]. For example, hereditary mutations of either the breast cancer (BRCA) 1 or the BRCA2 genes may increase the risk of developing breast or ovarian cancer [52,55,56] as the products of these genes are involved in the repair of double-stranded DNA breaks [57].

To conclude, cancer initiation occurs upon DNA damage, which is either not repaired or repaired incorrectly, and can result from either excessive DNA damage or a defective DNA maintenance system.

2.1.2. Promotion

In cancer, an initiated cell, may acquire mutations that confer survival and growth advantages to the cell that can drive clonal expansion via mitosis [42,58]. These initiated clones can survive indefinitely within normal tissues until re-stimulation/promotion [44] (Fig. 1). Furthermore, altered gene expression which favours increased cell numbers is a hallmark of cancer promotion (Fig. 3, sustaining proliferative signalling) [35,45,58].

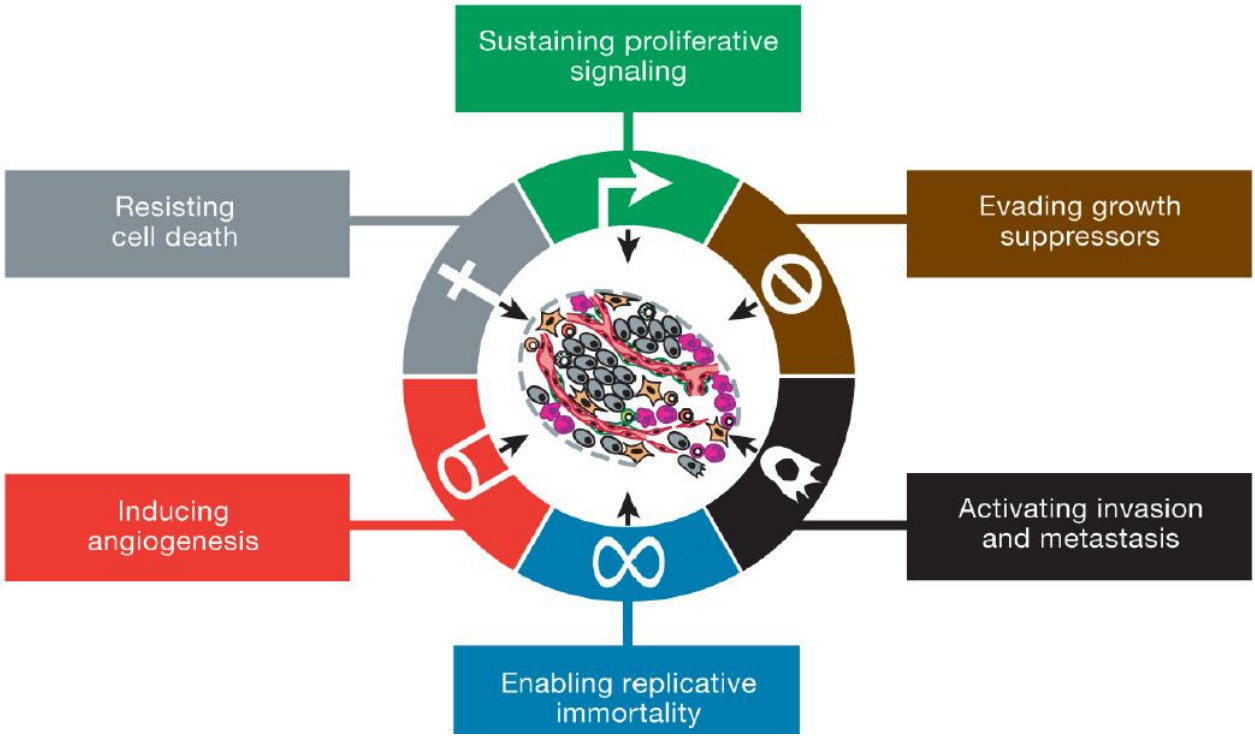

Figure 3. The six hallmarks of cancer. The hallmarks of cancer encompass six biological capabilities, resisting cell death, sustaining proliferative signalling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, and inducing angiogenesis, acquired during the various steps of tumour development. Taken from Hanahan *et al.* [35].

Various mutations have been identified that may contribute to the promotion of breast cancer. With regards to estrogen synthesis, mutations in the gene that produces aromatase, an enzyme crucial for estrogen synthesis [59], have been identified in breast cancers [60-62]. These mutations increase aromatase enzyme levels and, consequently, the *in situ* overproduction of estrogen, which acts as a growth factor in breast cancer tissues, may contribute to the growth of breast tumours [61,63]. Furthermore, estrogens exert their biological function at the target organ/tissue by binding to the estrogen receptor (ER), a member of the nuclear receptor family of transcription factors, which exists as two subtypes, ERα and ERβ [49,64-66]. The ER subtypes and their molecular mechanism of action will be discussed in detail in sections 2.2.1. and 2.2.2, but to clarify the significance of ER subtype mutations in breast cancer promotion it should be mentioned that the ERα subtype is associated with the stimulation of cell proliferation and the occurrence of breast cancer, while several studies have shown that ERβ ameliorates the action of ERα in breast cancer and could act as an inhibitor of breast cancer development [67-73]. An ERα gene mutation, Tyr537Asn mutation, has been identified that enables the transcription factor to activate its signalling pathway independently of ligand, thereby, sustaining proliferative signalling (Fig. 3), and this attribute may contribute to the hormone independent growth of tumours [74]. Also, amplification of the gene that encodes for ERα has been identified in proliferating breast cancers [75] and the elevation of receptor levels to create cells that are hyper-responsive to growth factors has been proposed as a mechanism of sustaining proliferative signalling [35]. Furthermore, a mutation in the ERβ gene that decreases ERβ expression has been identified and this decreased expression of ERβ may lead to an increased risk of breast cancer development [76]. Therefore, with regards to sustaining proliferative signalling in breast cancer, gene mutations that occur during initiation may influence growth factor production as well as their cognate receptors, thereby disrupting signalling pathways, resulting in the formation of actively proliferating tumour cells. Furthermore, although not discussed, it is important to note that a plethora of mutations [37,38,77-81] have been identified that may contribute to the promotion of cancer cells and that these mutations not only sustain

proliferative signalling (BRCA1 [82], cyclin D1 [38,78], CDK's [38], Myc [38]) but can also impact other hallmarks of cancer, such as evading growth suppressors and resisting cell death (p53 [37,38], Bcl-2 [37,38], Akt [38], PTEN [38]) [35,37,81].

2.1.3. Progression

The final step in cancer development is progression (Fig. 1) and during this step the initiated cells that obtained hallmark capabilities, such as sustaining proliferative signalling, evading growth suppressors, and resisting cell death, during promotion will undergo further growth and division to generate new highly proliferative clones with invasive and metastatic potential [42]. Furthermore, progression encompasses the three remaining hallmarks of cancer, namely enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (Fig. 3).

Cell senescence in normal cells is an irreversible process where cells become non-proliferative while maintaining metabolic activity and, furthermore, is inevitably followed by cell death [35,83]. Therefore senescence limits the replicative potential of a cell, thereby inhibiting uncontrolled proliferation. Telomeres are repeated sequences located at the end of chromosomes that shorten after every cell division and at a certain telomere length cells become senescent followed by apoptosis [84]. Therefore, the length of telomeres is implicated in acquiring replicative immortality [35,85]. Telomerase, an enzyme that adds telomeric repeats to telomeres, is expressed in low levels in normal cells but in high functional levels in cancer cells [86,87]. In normal cells telomerase counters erosion of telomeres due to factors other than cell division, however, in cancer cells the enzyme maintains telomeric length and thereby the cell avoids senescence and apoptosis [35,85]. Furthermore, it has been shown that estrogen, as a physiological stimulus, can increase telomerase activity in MCF-7 breast cancer cells and human ovary epithelium cells via the ER, thus implicating estrogen and its cognate receptor in avoidance of senescence and apoptosis to allow breast cancer cells to acquire immortality [88,89].

The cancer cell, which through initiation, promotion, and progression, has evolved into a tumour of immortal hyper-proliferating cells, like normal cells, requires nutrients and oxygen and must

remove carbon dioxide and metabolic waste [35,90]. The formation of new blood vessels or angiogenesis is employed by tumours to provide for these requirements [35,90,91]. Hypoxia, reduced oxygen availability, is a key signal for angiogenesis induction [91]. Studies have shown that nitric oxide (NO) production, and therefor hypoxia, is increased via estrogen activated ERα [92] and that angiogenesis may be inhibited by antiestrogens [93], establishing the role of estrogen and ERα in angiogenesis. Furthermore, a marker of hypoxia, hypoxia-inducible factor 1 (HIF-1), is considered a key initiator of angiogenesis in tumours [94] and increases during breast cancer development. HIF-1 is also associated with increased ER expression as estrogen stimulates a signalling pathway that may play a role in HIF-1 activation [94]. Therefore, estrogen en ERα may contribute to angiogenesis by either inducing hypoxia or by up-regulating initiators of angiogenesis and, thereby, play a role in sustaining developing tumours.

Tumour invasion and metastasis, the sixth and final hallmark of cancer, is a process whereby cancer cells from a primary tumour invade surrounding tissues and migrate to distant sites, thereby spreading the cancer through the body [35,95,96]. This final step in cancer progression comprises several rate limiting steps: (1) local invasion: invasion of adjacent tissues, (2) intravasation: cancer cells move into nearby blood and lymphatic vessels, (3) transit: cancer cells move through the lymphatic and blood systems, (4) extravasation: cancer cells move from lymphatic and blood vessels into distant tissues, (5) micrometastases: formation of small cancer nodules in distant organs, and (6) colonization: growth of macroscopic tumours in distant tissues [35,96,97]. Furthermore, if any of these steps fail the entire process may be inhibited [97], an attribute which could be exploited for therapeutic purposes. In breast cancer, metastasis at distant organs is the most common form of cancer re-occurrence and the foremost cause of fatalities [98,99], however, patients with ER positive tumours have a more favourable prognosis than patients with ER negative tumours [98]. Thus, with regards to breast cancer invasion and metastasis this suggests a protective role for the ERs. Epithelial to mesenchymal transition (EMT) promotes cancer invasion during breast cancer progression and is characterized by a loss of cellular adhesion [100-102]. Nuclear

factor-kappa B (NFκB), a pro-inflammatory transcription factor, is involved in the development of breast cancer [47,103,104]. NFκB consists of five subunits, NF-κB1, NF-κB2, RelA/p65, RelB and c-Rel [105], of which the RelB subunit has been shown to induce the expression of Bcl-2, an inhibitor of apoptosis that promotes cell survival and induces EMT in human mammary epithelial cells [105-107]. Furthermore, ERα inhibits RelB synthesis, thereby inhibiting Bc-l2 expresion and therefore, inhibiting EMT [108]. In addition, both ER subtypes have been shown to inhibit EMT by inducing expression of E-cadherin, an epithelial marker and adhesion molecule [100,109].

Thus far I have discussed the development and progression of cancer, highlighting some of the situations where estrogens and their cognate receptors may influence breast cancer development and progression. The steps of cancer development (Fig. 1), as well as the hallmark capabilities of cancer (Fig. 3), present several possible therapeutic targets for current breast cancer treatment as well as the development of new treatments. Before I discuss these treatments, I will elaborate on the estrogens, the ER and its subtypes, ERα and ERβ, and how they may regulate signalling pathways that can contribute to breast cancer development and progression.

2.2. Estrogen and its molecular mechanism of action

Estrogens are pleiotropic hormones [110] traditionally linked to the development of the female reproductive tract and secondary female sex characteristics [48-50,111] and can affect cell viability, cell proliferation, and gene expression [17,112,113]. Furthermore, estrogens also regulate cardiovascular physiology, bone integrity and neuronal growth [111]. In pre-menopausal women more than 95% of these estrogens are synthesised in and secreted by the ovary with the remainder being synthesised from steroid precursor molecules in tissues such as, for example, breast and adipose tissues [59,114,115]. In contrast, in post-menopausal women estrogens are predominantly produced by the peripheral conversion of steroid precursor molecules [116]. In brief, estrogen steroidogenesis (Fig. 4) entails the conversion of cholesterol to pregnenolone by the CYP11A1 enzyme, where after pregnenolone is hydroxylated by CYP17A1 to form 17OH-pregnenolone, which in turn is converted to dehydroepiandrosterone (DHEA) by the same enzyme [117,118].

DHEA is converted to either androstenedione by 3β-hydroxysteroid dehydrogenase/isomerase type 2 (3βHSD2) or androstenediol by 17β-hydroxysteroid dehydrogenase/isomerase type 1 (17βHSD1) [117].

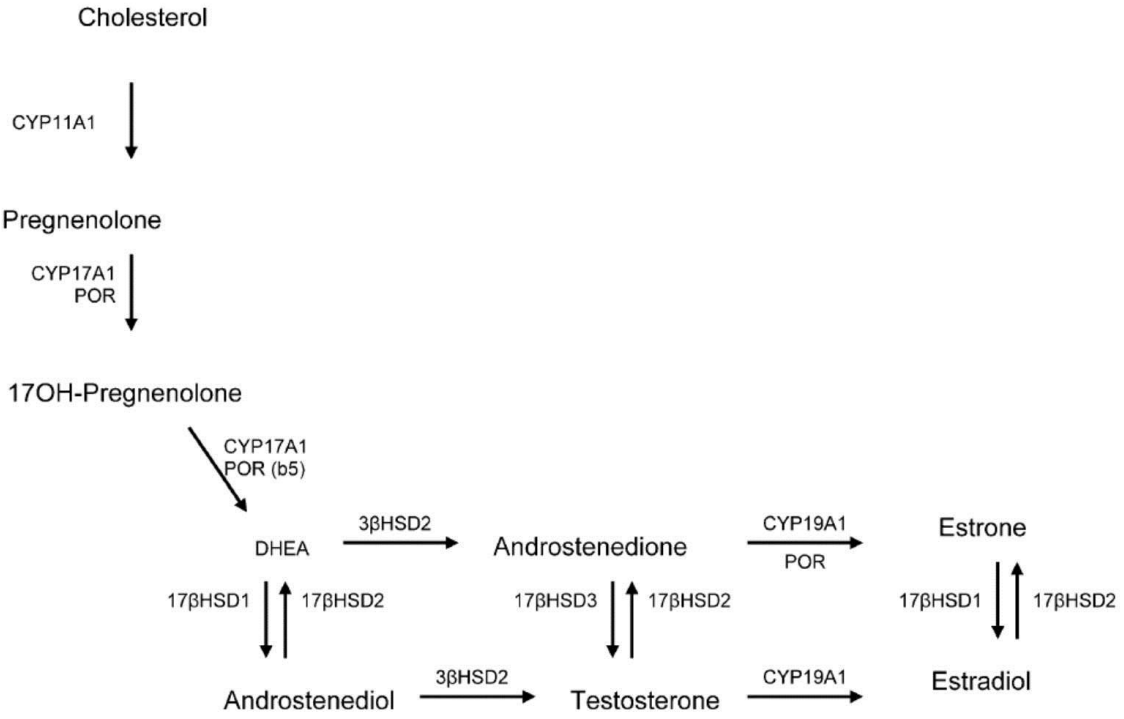

Figure 4. Conventional pathway of estrogen steroidogenesis. Cholesterol is converted to estrone and estradiol through a series of enzyme regulated reactions. 3βHSD, 3β-hydroxysteroid dehydrogenase/isomerase; 17βHSD, 17β-hydroxysteroid dehydrogenase/isomerase; b5, Cytochrome b5; CYP17A1, Cytochrome P450 17A1; CYP19A1, Cytochrome P450 19A1; POR, Cytochrome P450 reductase. Figure taken and adapted from Ghayee *et al.* [117].

Androstenedione can either be converted to testosterone by 17βHSD3 or E_1 by CYP19A1 (aromatase enzyme), whereas androstenediol is converted to testosterone by 3βHSD2, which in turn is converted to E_2 by CYP19A1 [117-119]. The weak estrogen, E_1, estrone, is converted to the potent and biologically active E_2, estradiol, by 17βHSD1 [117]. Once synthesised/secreted the estrogens are transported to target organs by a plasma glycoprotein known as sex hormone binding globulin (SHBG) which, in turn, also regulates the availability of estrogens to the target organs [120-122]. At these target organs, such as the uterus, vagina, liver, bone, and breast [110,123-125], estrogens are released from SHBG, diffuse across the cell membrane and bind to mostly nuclear estrogen receptors (ERs) [64,126,127].

2.2.1. Estrogen receptors

Estrogen receptors form part of the nuclear receptor family of transcription factors, a family that shares a similar structural homology and represents the largest family of transcription factors [49,65]. Up until 1996 estrogens were thought to elicit their action through only one ER subtype (renamed ERα after discovery of second subtype) but this changed in 1996 with the discovery of a second subtype, ERβ [128,129]. These receptor subtypes are produced by different genes, located on different chromosomes and therefore ERβ is not formed by differential splicing of ERα [50].

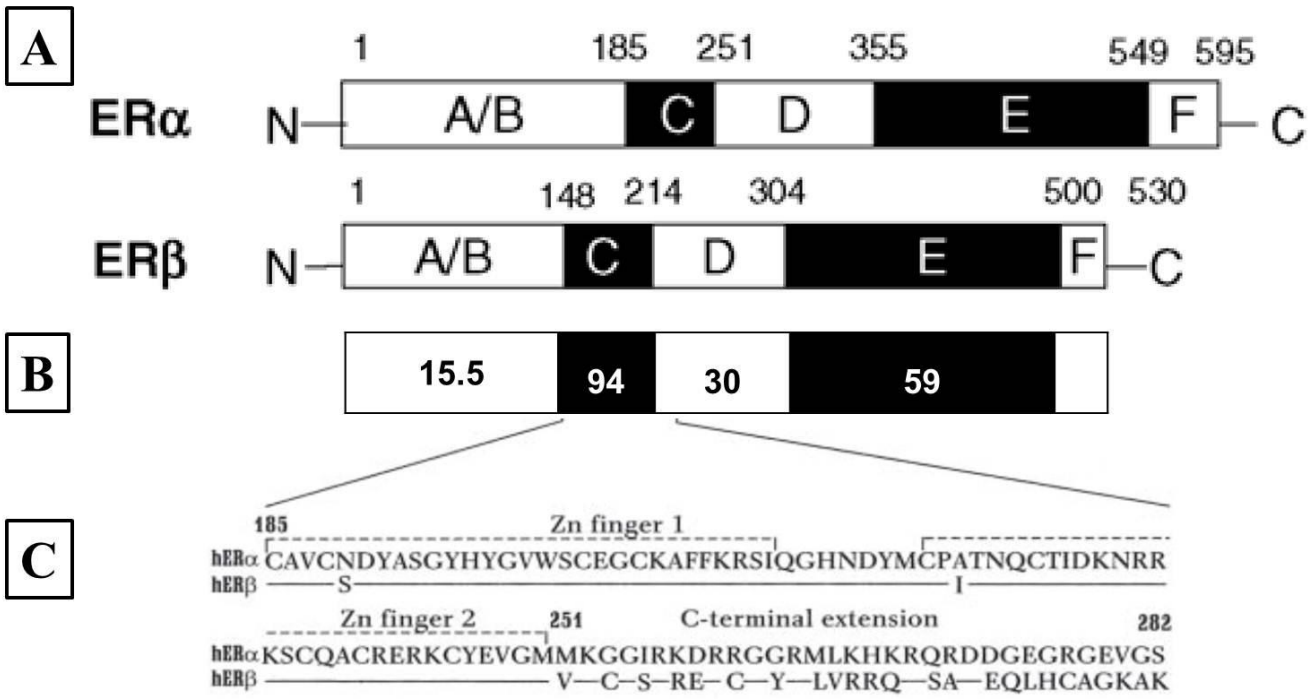

Figure 5. Human estrogen receptors are comprised of two subtypes, ERα and ERβ. (A) Schematic representation of the functional domain organization of the human ERs, (B) the percentage identity of the domains of human ERβ with human ERα, and (C) the amino acid sequences of the ERα and ERβ DNA binding domains (only divergent amino acids are indicated for ERβ). A/B, N-terminal region, which contains AF-1; C, DNA binding domain; D, hinge region; E, ligand binding domain, which also contains AF-2; F, C-terminal region. Adapted from Ruff *et al.* [130], Beck *et al.*[131], Enmark *et al.*[50] and Sanchez *et al.* [127].

Both receptor subtypes, shown in Fig. 5, consists of homologous regions known as domains and the three major domains are the ligand binding domain (LBD), the DNA binding domain (DBD), and the activation domains (AF) [49].

These homologous domains have different functions that confer specificity for binding of the ER to the estrogen response element (ERE) in the promoter regions of estrogen responsive genes [65,132].

The DBD (Fig. 5C) consists of approximately 70 amino acids that are folded into two zinc-finger motifs that are highly conserved between the receptor subtypes. Furthermore, within these zinc-finger motifs, two perpendicular α-helices extend from the base of the fingers, both of which have distinct functions [50,65]. The first helix interacts with DNA and conveys specificity, whereas the second finger has a dimer interface that directs subunit interactions for receptor dimerization [50,65,133-135]. The LBD (Fig. 5), which confers specificity for ligand binding and performs functions associated with ligand binding such as receptor release from heat shock proteins (HSPs), dimerization, interaction with co-activators as well as co-repressors, and transcriptional activation [65,127,130], is less conserved between the ER subtypes, a characteristic reflected by the diverse structures of ligands bound by these receptor subtypes [127]. Despite of the lower percentage homology of the LBD of the ER subtypes, 17β-estradiol (E_2) has similar binding affinities for the two ER subtypes [50]. Furthermore, apart from the DBD and the LBD, each receptor subtype has an N-terminal domain which contains the transcription activation function 1 (AF1) [65,127] which, along with AF2 in the LBD, are associated with the recruitment of co-activators [50,136]. There is considerable variation between the ER subtype AF1 domains, which has a ligand-independent transactivation function, whereas AF2, the ligand dependent transactivation domain, is more conserved [137]. Variation in the in the AF1 domain may account for the high ligand independent transcriptional activity of the ERβ subtype [138]. The ERs also contain a flexible hinge region (Fig. 5), which is less conserved between the ER subtypes, and this region contains nuclear localization signal information and plays a role in orientating the AF1 and AF2 domain for optimal E_2-driven transactivation [137].

Physiologically, while ERα is associated with the promotion of cell proliferation that contributes to the occurrence of breast and endometrial cancer, several studies have shown that ERβ inhibits ERα-dependent cell proliferation and could prevent cancer development [67-73,139,140]. Furthermore, the subtypes stimulate the transcription of both common and distinct subsets of E_2 target genes [69,110,141,142]. However, in many cases the degree of activation via ERβ is lower [50], despite

the high ligand independent transcriptional activity of this subtype [138,143]. In addition, in breast cancer, higher ERα levels are associated with malignant tumours, while higher ERβ levels are associated with benign tumours [72,144]. Having discussed the synthesis of estrogen, as well as the receptors through which it elicits its function, the next section will elucidate the mechanisms whereby the ER elicits its function.

2.2.2. Molecular mechanisms of action of the estrogen receptor.

Unliganded ERs are mainly located in the nucleus of target cells [65,126,127] in an inactive complex consisting of the ER, HSPs and immunophils [127,145,146]. Classically (Fig. 6-1), upon binding of E_2 to the ER, the activated ER will dissociate from the chaperone complex (HSPs and immunophils), undergo a conformational change, dimerize, and bind to EREs in the promoter regions of target genes [17,49,65].

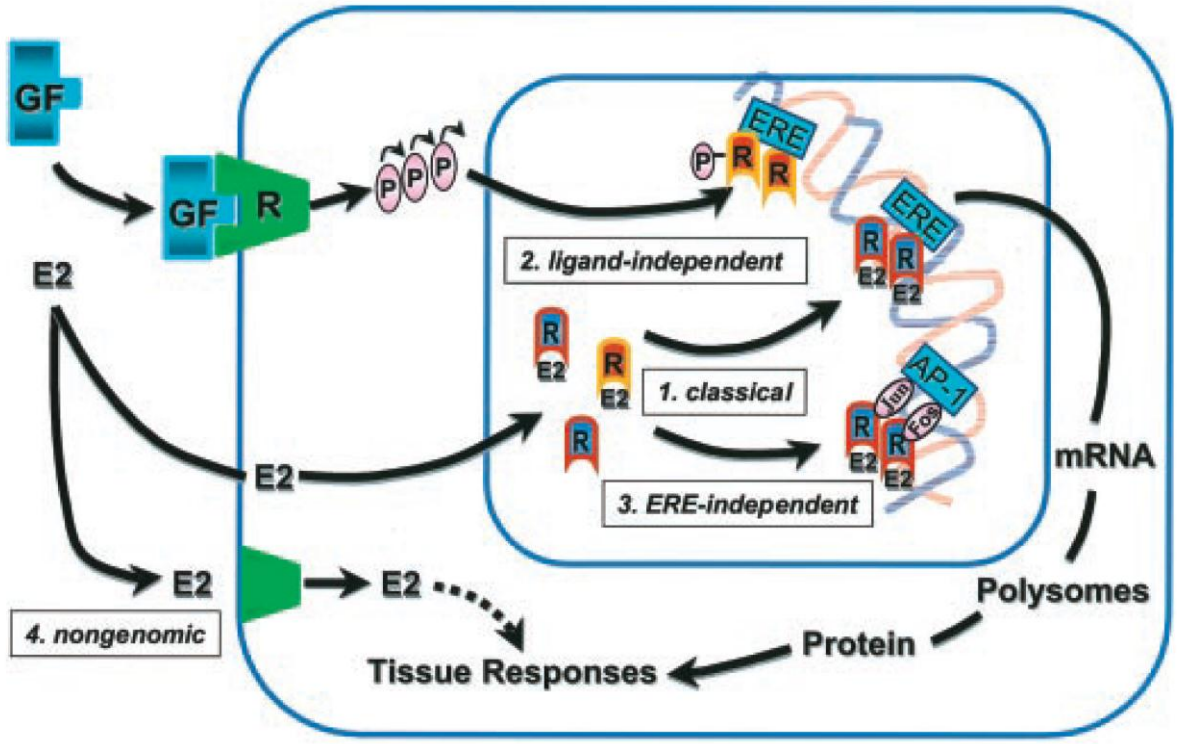

Figure 6. Classic and alternative models of estrogen mediated transactivation. Upon estrogen (E_2) binding the ER can activate gene transcription via (1) classical DNA binding or (3) tethering to DNA bound transcription factors. Furthermore, (2) unliganded phosphorylated ER can directly bind to DNA or (4) membrane bound-ERs can elicit a nongenomic effect. Figure taken from Hall *et al.* [147]. GF, growth factor; R, receptor; ERE, estrogen response element; AP-1, activating protein-1.

However, the ER can also activate gene transcription by implementing non-classical pathways (Fig. 6-3) such as for activating protein-1 (AP-1), where the activated ER does not directly interact with the DNA in the promoter regions of AP-1 regulated genes, but tethers to the already bound transcription factors (c-Jun and c-Fos) and thereby influences transcription [148-151]. Furthermore, ER function may also be modulated by extracellular signals in the absence of E_2 (Fig. 6-2) where the ER is activated via phosphorylation by, for example, signalling pathways initiated by epidermal growth factor (EGF) or insulin-like growth factor (IGF-1) [147,152,153]. Lastly, membrane bound-ERs (Fig. 6-4) have been identified and it has been shown that they are G protein-linked and can elicit a range of signal transduction events like, for example, the induction of cell proliferation and the inhibition of apoptosis [147,154-157]. In addition, the intracellular ER may also be localised at the cell membrane and activate signal transduction pathways such as the MAP-kinase pathways [156,158].

The two ER subtypes, ERα and ERβ, introduce another level of complexity to the molecular mechanism of estrogen action via the ERs. These subtypes may dimerize to form ERα/α homodimers, ERβ/β homodimers, or ERα/β heterodimers [159-161]. All three dimer pairs can bind to DNA and are transcriptionally active, but with varying degrees of activity [143,162,163]. It is presumed that ERα/α homodimers increase cell proliferation and thereby contribute to cancer development, whereas the ERβ/β homodimer has an opposing protective effect [69,162-164]. Furthermore, it has been suggested that the ERα/β heterodimer activates genes that are distinct from those activated by either homodimer and that the heterodimer has a growth inhibitory effect on breast epithelial cells [160,162,165]. Therefore, dimerization, both homo- and heterodimerization, is an important characteristic of the ERs that may impact the development and progression of breast cancer.

In a previous section it was mentioned that NFκB, a pro-inflammatory transcription factor, is involved in the development of breast cancer [47,103,104] and therefore, it is important to state that liganded ER not only activates gene transcription but can also repress it. For transrepression,

specifically the repression of NFκB driven genes, various mechanisms of ER mediated transrepression have been described [166]. The ER may bind to NFκB and thereby prevent DNA binding of the transcription factor [167,168], ligand bound ER present at promoter regions may recruit co-repressors [169,170], ligand bound ERα and activated NFκB may compete for co-activator recruitment [171,172], or ERα, through a non-genomic pathway, may inhibit translocation of activated NFκB to the nucleus [173]. Furthermore, it has also been suggested that the ER may inhibit production of interleukin-6 (IL-6), a pro-inflammatory cytokine, not only by transrepression of NFκB, but also by transrepression of CCAAT/enhancer-binding protein beta (C/EBPβ) activity via a protein-protein interaction [174]. In addition, ERα may inhibit matrix metalloproteinase-1 (MMP-1) transcription by tethering to AP-1 sites in the promoter region of the MMP1 gene [175]. Although ERα has been shown to inhibit transcription by tethering to an AP-1 site, estrogen may also activate transcription from some AP-1 sites via ERα, whereas it inhibits this activation via ERβ, while, in contrast, antiestrogens may activate transcription from these AP-1 sites via ERβ [176]. Therefore, these findings further illustrate the complexity of estrogen signalling via the ER.

To conclude, estrogens are sex hormones traditionally linked to female reproductive development and exert their function via the ER, which exists as two subtypes, ERα and ERβ. Furthermore the ERs may activate or repress the transcription of genes by various mechanisms. Also, earlier we discussed how estrogens may contribute towards the development and progression of breast cancer. Therefore, in the following section we will discuss how estrogen and its cognate receptors are targeted for the treatment of breast cancer at a molecular level.

2.3. Treatment of breast cancer at the molecular level.

Currently, endocrine responsive breast cancer, both ER positive and hormone dependent [177], which accounts for approximately 75% of breast cancers, is treated by the administration of adjuvant endocrine therapy (ET) in the form of aromatase inhibitors (AIs), ovarian function suppression, or the selective estrogen receptor modulators (SERMs), like tamoxifen [116,178-180]. Blocking of estrogen production by AIs is an established therapeutic option for post-menopausal

women where estrogens are mainly produced from the peripheral conversion of androgens by aromatase [116,181-183], however, this is not an effective therapeutic option for pre-menopausal breast cancer patients as pre-menopausal estrogen levels cannot be efficiently reduced [184,185]. Aromatase inhibitors inhibit the CYP19A1 aromatase enzyme (Fig. 4) that coverts androgens to E_1 and E_2 with the goal of reducing the circulating levels of estrogens [181]. Current AIs may be divided into two groups, the steroidal, type 1, and non-steroidal, type 2, AIs [116,182,186]. The steroidal AIs have a steroidal structure similar to that of the substrate of the aromatase enzyme which, upon binding, is converted to an intermediate that covalently binds to the enzyme, thereby irreversibly inactivating the aromatase enzyme [116,186]. In contrast, the non-steroidal AIs prevent androgen binding by non-covalently binding to the aromatase enzyme and saturating the binding sites [116]. Furthermore, although AIs are successfully used for the treatment of endocrine responsive breast cancers and are mostly associated with only mild side effects such as hot flashes [187-189], joint pain [187,190], vaginal dryness [189] and headaches [187], their use is hampered by a significant increase in the occurrence of osteoporosis [116,187,191] and AI resistance in a considerable number of patients [116,192]. In addition, the use of AIs is not an effective therapeutic option for pre-menopausal breast cancer patients as AIs alone cannot efficiently reduce pre-menopausal estrogen levels [184,185]. Thus, for these patients complete ablation of ovarian function by either surgical oophorectomy [193,194], radiation induced ablation [195], the use of luteinizing hormone- or gonadotropin hormone-releasing hormone (LHRH or GnRH) agonists [185,195,196], or chemotherapy [197] is an option [183,184]. Furthermore, although both the above mentioned treatments, with estrogen levels as a target, is effective for the treatment of breast cancer it is accompanied by undesirable side-effects such as adverse menopausal symptoms and the increased occurrence of osteoporosis. Therefore, the development of breast cancer therapies is on-going and another possible target for these therapies is the ERs.

The search for new and improved breast cancer therapies that target the ERs heralded the era of the SERMs with selective ER modulation being described for the first time in 1987 [198]. SERMs are

compounds that, like estrogen, can interact with the ER but have tissue specific activities that differ from that of estrogen [198-202]. Ideally, a SERM would act as an ER antagonist in the breast, thereby antagonizing the proliferative effect of E_2 via the ER, and act as an ER agonist in the bone, thereby preventing osteoporosis associated with decreased levels of estrogen. Several SERMs have been developed with the following proposed molecular mechanisms of antagonist action: SERMs may act as antagonists by binding to the ER with a higher affinity than E_2 and block the binding of E_2, they may block the binding of co-activators, or SERMs may induce the recruitment of co-repressors. [203-205]. Not much is known regarding the molecular mechanism of SERM agonism [203], although it has been suggested that they may block the binding of co-repressors [205] or activate cell surface signalling pathways that result in ligand-independent activation of ERs [147,199,206,207].

Tamoxifen, a first generation SERM and one of the most commonly used ETs for breast cancer treatment, fulfills the requirement of being an ER antagonist in breast tissue but only increases bone mineral density in postmenopausal, not pre-menopausal, women with breast cancer [202,208,209]. However, not only has tamoxifen usage been linked to an increased risk of venous thromboembolism and occurrence of hot flashes, but it also acts as an agonist in the endometrium and thereby stimulates endometrial growth and endometrial hyperplasia, a risk factor for endometrial cancer [199,210-213]. The adverse tamoxifen associated side-effects instigated the development of further generations of SERMs. The second generation SERM, raloxifene, is an improvement from tamoxifen as it is an antagonist in both the breast and the endometrium, while being an agonist in bone tissue [209,214-216], however it does increase the risk of venous thromboembolic diseases [199,210]. Third generation SERMs, such as lasoxifene and bazedoxifene, are currently in development, however, the focus of the research endeavor has shifted to osteoporosis treatment with protection against breast cancer as a beneficial side effect [198,201,217].

SERMs bind to the ER and affect estrogen signaling thus modulating breast cancer development and progression, however, the down-regulation of ER protein levels could also attenuate the effect of excessive estrogen levels. The prospect of down-regulating ER levels for therapeutic purposes instigated the development of selective estrogens receptor down-regulators (SERDs). Natural estrogens down-regulate protein levels of both of the ER subtypes by enhancing ER ubiquination for consequent ubiquitin-proteasome pathway mediated degradation [218-225]. However, the ideal SERD would only down-regulate ERα levels, associated with cell proliferation and cancer development, while stabilizing, or up-regulating, ERβ levels, which inhibits ERα-dependent cell proliferation. Fulvestrant, also known as Faslodex or ICI 182,780, is a complete ER antagonist that down-regulates ERα levels [218,226] while stabilizing ERβ protein levels [218]. Furthermore, fulvestrant has been shown to inhibit the growth of breast tumour xenografts [227,228]. During clinical trials, fulvestrant treatment presented undesirable, yet minor, side effects such as headaches, hot flushes, nausea, and disturbance of menses [229]. However, the poor bioavailability as well as length of time required to achieve a useful therapeutic concentration in target tissues, weighs against fulvestrant as an ideal breast cancer treatment [227,230]. GW5638/DPC974, an orally active non-steroidal tamoxifen derivative and SERD [227,230], also down-regulates ERα levels [230,231]. Further development of GW5638/DPC974 was discontinued but afore mentioned findings merits further investigation regarding SERDs.

All of the discussed treatments (AIs, SERMs, and SERDs) display beneficial properties for the prevention and treatment of breast cancer, although, through either targeting estrogen synthesis or the ER, undesirable side-effects are still a problem. Interestingly many of these side-effects are also side-effects that are associated with the depletion of estrogen during menopause, such as hot flushes, night sweats, sleeping problems, vaginal dryness [2,4] as well as an increased risk of osteoporosis [5]. Therefore, it may be beneficial to find a treatment that not only inhibits breast cancer development and progression, but also alleviates menopause associated side effects. It could be postulated that to achieve this goal you would require a therapeutic agent that could mimic the

beneficial effects of estrogen in some contexts, while inhibiting estrogen associated proliferation in the breast and uterus. Clearly this would argue against an agent that is a complete anti-estrogen or results in blocking estrogen synthesis.

Figure 7 delineates the beneficial as well as unfavourable characteristics of current HRT and the current SERMs, tamoxifen and raloxifene, while highlighting the properties of an ideal SERM.

Previously I discussed the opposing roles of the ER subtypes in breast cancer development, where ERα is proliferative and ERβ is anti-proliferative. These opposing characteristics of the ER subtypes has guided the search for an effective therapeutic compound that could alleviate menopausal symptoms while preventing breast cancer development and progression towards compounds that target the ER subtypes. These compounds would not be SERMS, but rather selective estrogen receptor subtype modulators (SERSMs). The ideal SERSM would be an ERα selective antagonist [232], while selectively activating ERβ transcriptional pathways [139,140,233,234], would down-regulate ERα protein levels while stabilizing ERβ protein levels [232,235] and would display anti-inflammatory properties by inhibiting transcription of pro-inflammatory genes to prevent the occurrence of post-menopausal osteoporosis [139,236]. Advances towards finding the elusive ideal SERSM have been made and several subtype specific compounds have been identified, for example methyl-piperidino-pyrazole (MPP) (ERα specific antagonist) [237,238], diarylpropionitrile (DPN) (ERβ specific agonist) [239], ERB-041 (ERβ specific agonist) [240,241], liquiritigenin (ERβ specific agonist) [234], which was isolated from the plant extract MF101 (ERβ specific agonist) [233] and the *R, R* enantiomer of 5,11-*cis*-diethyl-5,6,11,12-tetrahydrochrysene-2,8-diol (THC, ERα agonist and ERβ antagonist) [242].

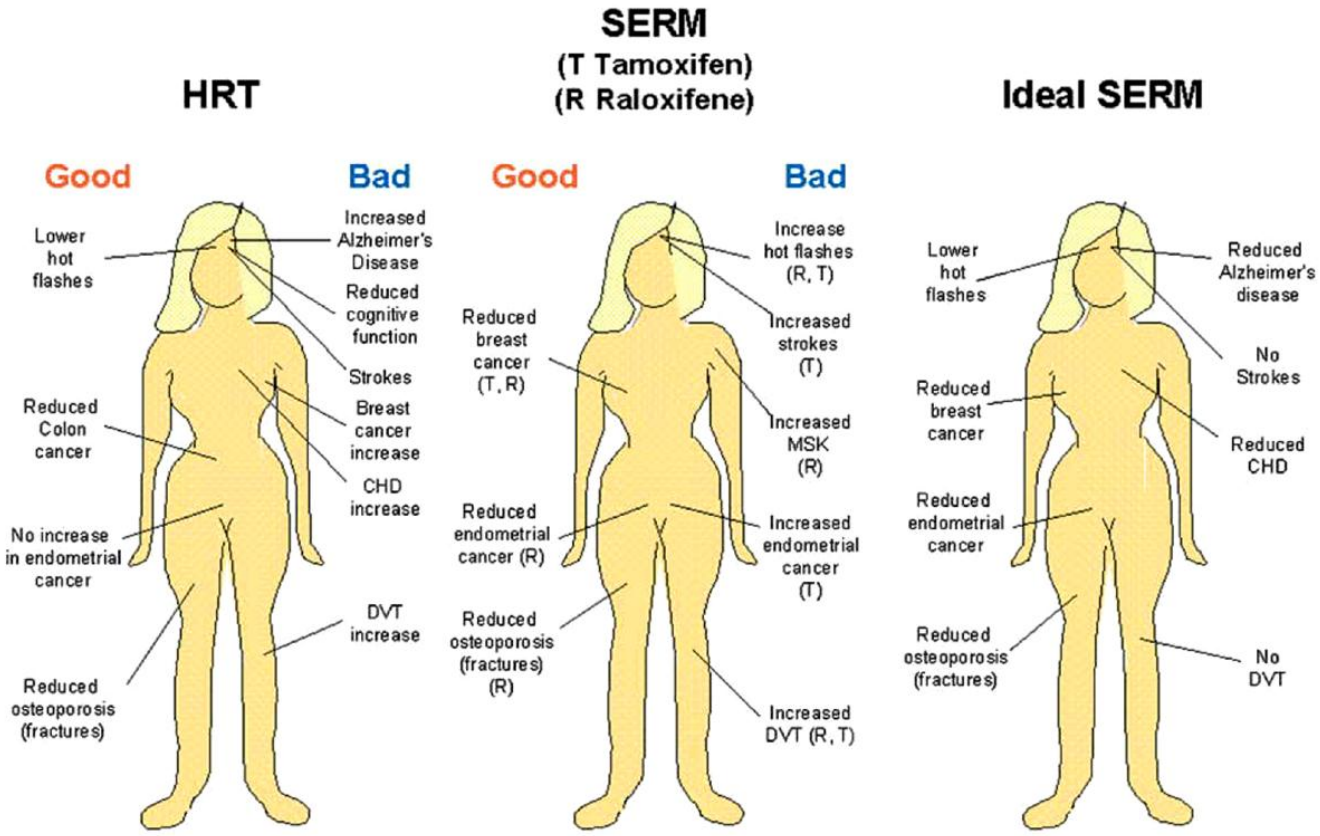

Figure 7. Development towards finding the ideal selective estrogen receptor modulator (SERM). A summary of the good and bad effects of current hormone replacement therapy (HRT), the benefits and shortfalls of the known SERMs, tamoxifen (T) and raloxifene (R), and the characteristics of an ideal SERM. CHD, coronary heart disease; DVT, deep vein thrombosis; MSK, musculoskeletal side effects. Figure taken from Jordan, V.C., 2004 [243].

The isolation of a highly specific ERβ agonist, liquiritigenin, from a plant extract, MF101, which itself is an ERβ agonist, raises the question of whether the ideal SERSM may be found in plant material? This question guides us towards phytoestrogens, plant compounds that are referred to as natural SERMs and which may be both estrogenic as well as anti-estrogenic [244-247]. Despite conflicting evidence regarding doses of phytoestrogens and breast cancer risk [248,249], findings regarding the subtype specificity of phytoestrogens [250-252] have generally pointed the search in the direction of phytoestrogens and focused attention on phytoestrogen rich food sources as a possible source of the ideal SERSM. Furthermore, reluctance among concerned users regarding the use of "unnatural" substances, has led to a search for safer and more "natural" products as an alternative to synthetic drugs [253,254].

2.4. Phytoestrogens

The occurrence of breast cancer in Asia is much lower compared to that of Western countries [255-257]. However, in countries like Japan the occurrence of breast cancer is increasing and one of the proposed contributing factors is the change to a more "Western" diet consisting of more food from animal origin [256]. Furthermore, the low incidence of hormone dependent cancers, such as breast cancer, among these populations has been proposed to be the result of diets rich in, for example, soybean products, which contain phytoestrogens [255,258]. These differences in breast cancer occurrence focused attention on phytoestrogens and their use as chemo-preventive substances.

As previously mentioned, phytoestrogens are non-steroidal compounds from plant origin with the ability to have estrogenic or anti-estrogenic properties [259]. Phytoestrogens have a 2-phenylnaphthalene-type chemical structure similar to that of estrogen (Fig. 8) and may bind to both of the estrogen receptor subtypes [260]. Phytoestrogens may be divided into three major classes, the isoflavones, coumestans, and lignans [259,261]. The isoflavones, genistein, daidzein, and glycitein, are the major phytoestrogens that may be obtained via dietary intake and their main sources are legumes and soy products [261,262], while coumestans, like coumestrol, are found in bean sprouts and clover [262]. Furthermore, the lignans, enterodiol and enterolactone, are referred to as mammalian lignans as they are produced by the gut microflora in the colon from plant lignan precursors, found in, for example, linseed, wheat, and bran [262-265].

The isoflavone, genistein, is well studied and can bind to both ER subtypes although it generally has a higher affinity for ERβ [250-252,266]. Furthermore, genistein exerts an estrogenic effect through both of the ER subtypes and can recruit co-regulators for the transcription of ERα and ERβ selective genes [131,250,267]. Also, genistein inhibits proliferation of ER positive MCF-7 cells, a human breast cancer cell line, both in the presence [252,268] and absence [269] of E_2 and this effect is mediated via the ER [252,269]. In addition, perinatal exposure of rats to physiologically relevant concentrations of genistein provides protection against chemically induced mammary cancer

development [270] and, furthermore, this early exposure to genistein promotes the differentiation of mammary cells which, in adulthood, may suppress the formation of breast cancer [270-272].

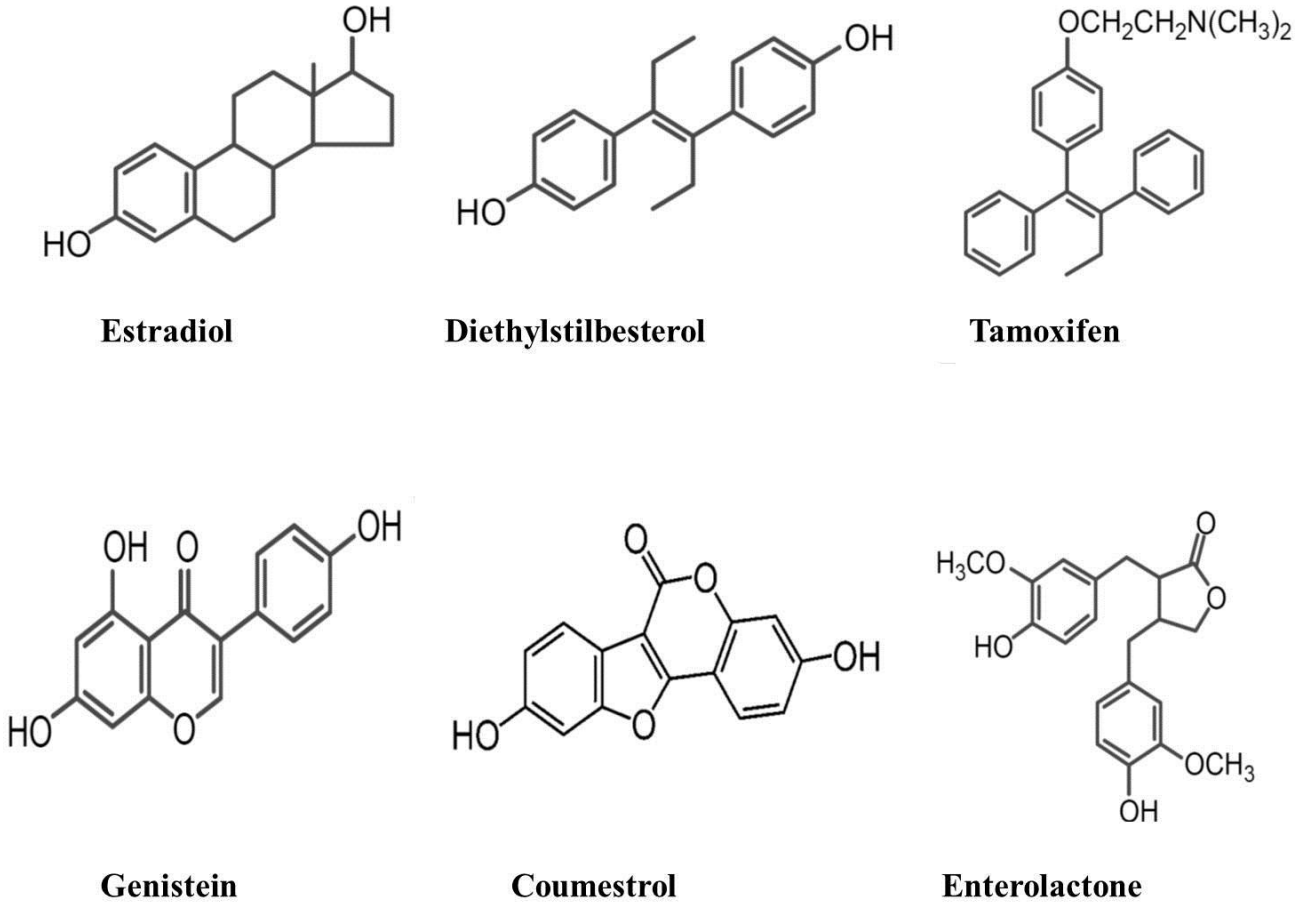

Figure 8. Structures of examples of the major classes of phytoestrogens. Structures of genistein (isoflavone), coumestrol (coumestan), and enterolactone (lignan) compared to the endogenous estrogen (estradiol), a synthetic estrogen (diethylstilbesterol), and a SERM (tamoxifen, a synthetic estrogen). Structures taken from Duncan *et al.* [259] and Kshirsagar *et al.* [273].

Genistein has also been shown to inhibit angiogenesis, have anti-oxidant properties, and decreases rat ovarian and rat uterine aromatase activity and therefore, may inhibit both cancer initiation as well as progression [259,261,274-276]. With regards to menopause and HRT, isoflavones attenuate bone loss in perimenopausal women [277] and may reduce hot flashes [278,279]. Although genistein has many positive attributes regarding breast cancer there are cautionary findings, such as, that neonatal exposure to genistein in the physiological range induces uterine adenocarcinoma [280], that low concentrations of genistein induces MCF-7 cell proliferation [281], that genistein

can inhibit MCF-7 cell apoptosis [282], and that exposure to genistein after the development of an estrogen dependent tumor will promote tumor growth [281].

The mammalian lignans, enterodiol and enterolactone, inhibit MCF-7 cell proliferation more pronouncedly than in ER negative breast cancer cells [283], decrease breast cancer cell viability in the presence of E_2 [284], and reduce MCF-7 tumor xenograft growth and angiogenesis in mice [285]. Furthermore, both of the lignans can inhibit estrogen production via the aromatase and 17βHSD pathway and consequently reduce cell proliferation [286]. In addition, enterolactone has a low binding affininty for both of the ER subtypes [266], but can transactivate estrogen dependent gene transcription, albeit with a low potency [266,284]. Therefore the mammalian lignans, produced from plant pre-cursors, show potential as chemo-preventative as well as chemotherapeutic compounds.

Coumestrol, the major coumestan [262,287], is not as well studied but has been shown to bind to the ER [288,289], have higher estrogenic potency than genistein [290], transactivate gene transcription through both ER subtypes with stronger activation through ERβ [267], induce MCF-7 cell growth [282,291,292], and inhibit breast cancer cell invasion without affecting cell viability [293]. Therefore, although coumestrol can inhibit breast cancer cell invasion and thus inhibit the progression of breast cancer, caution is advised for use with estrogen dependent tumors. However, the use of phytoestrogenic coumestrol as an alternative to traditional HRT is a possibility that may still be explored.

Phytoestrogens may be referred to as natural SERMs as they have been shown to bind to the ER and have tissue specific estrogenic and anti-estrogenic properties [244,294]. For example, genistein has been shown be anti-estrogenic in breast tissue and cells [252,269,270] and estrogenic in bone tissue [294] and the uterus [295]. In addition phytoestrogens have also been shown to have selective ER subtype modulating properties. Genistein can bind to both ER subtypes but displays a higher affinity for ERβ [250-252], whereas genistein as well as coumestrol, although not being ER subtype selective, display stronger transactivation of gene transcription via ERβ than via ERα [252,267]. In

addition, the phytoestrogen, liquiritigenin as well as the plant extract, MF101, are ERβ specific agonists [233,234]. Taken together these findings suggest that an ER subtype specific modulator may be found in phytoestrogen containing plant material.

In conclusion, phytoestrogens generally show potential to be developed for the treatment of menopause associated side effects as well as for breast cancer chemo-prevention. However, contradictory findings regarding the treatment of hormone sensitive cancers highlights the importance of further research, especially research that will determine the optimal time of treatment during the development and progression of cancer. Despite these contradictory findings, the general ER subtype specificity of phytoestrogens marks phytoestrogen rich food sources as an attractive target for finding the elusive ideal SERSM. One such source may be *Cyclopia*.

2.5. *Cyclopia*

Cyclopia (family: Fabaceae; tribe Podalyrieae), is an indigenous fynbos plant (Fig. 9) from the Western Cape province of South Africa [296,297]. Traditionally, the "fermented" (oxidized) form of *Cyclopia*, has been consumed as a fragrant, caffeine free honeybush tea beverage with the "unfermented" form being introduced to the commercial market more recently [252,297,298]. Anecdotal evidence associates the consumption of honeybush tea with being an appetite enhancer, a stimulator of milk production in breast feeding women, a treatment for a spastic colon, and it is believed to provide relief to arthritis sufferers [297]. Furthermore anecdotal findings suggest that honeybush tea alleviates menopausal symptoms [251]. These anecdotal findings instigated research into the beneficial health properties of *Cyclopia*, as well as, into identifying the compounds within *Cyclopia* that elicit these effects.

Previous studies have shown that extracts of various *Cyclopia* species increased the antioxidant status of the liver in rats [299], inhibited tumour promotion in mouse skin [300], inhibited aflatoxin B_1 (AFB_1) induced mutagenesis [301], provided protection against fumonisin B_1 (FB_1)-induced cancer in rat livers [302], inhibited esophageal cancer development in rats [303], and displayed anti-obesity characteristics by inducing lipolysis [304] and inhibiting adipogenesis [305]. The current

study, however, will focus on the phytoestrogenic properties of *Cyclopia* and the modulation of molecular targets involved in the prevention and treatment of breast cancer by the extracts of *Cyclopia*. Therefore I will limit my discussion to the phytoestrogenic properties of *Cyclopia* extracts and the polyphenols they contain.

Studies investigating the chemical composition of *Cyclopia* have shown that phenolic compounds with estrogenic activity, for example luteolin, eriodictyol, naringenin, and formononetin, are present in various species of *Cyclopia* [252,298,306-309]. Louw *et al.* (Addendum A, [308]) summarizes the known polyphenol content of *Cyclopia* and highlights the polyphenols present in the shoots and leaves of *Cyclopia* that have been shown to have phytoestrogenic properties. Furthermore it provides an overview of the potential uses of phytoestrogens and, in addition, states the importance of the standardisation of both the levels of active compounds as well as the activity levels (potency and efficacy) of these compounds with regards to the use of plant derived supplements for health benefits.

Figure 9. The *Cyclopia* plant. The shoots of two of the *Cyclopia* species, *C. subternata* (left) and *C. genistoides* (right). The yellow flowers are distinctive of the *Cyclopia* species. Photos taken from Louw *et al.* [308].

For the benefit of the reader I will briefly highlight certain points from Louw *et al.* (Addendum A, [308]) regarding the phytoestrogenic extracts of *C. genistoides* and *C. subternata* as they are relevant to this study.

P104, a dried methanol extract (DME) of *C. genistoides*, has been shown to bind with a lower affinity than E_2 to both of the ER subtypes [251,252], but with a higher affinity for ERα [252]. However, although having a higher affinity for ERα, P104 could only transactivate an ERE-containing promoter reporter construct via ERβ with an efficacy similar to that of E_2 but with a lower potency [252]. Furthermore, P104 induced MCF-7BUS cell proliferation with a lower potency than that of E_2, an effect shown to be mediated via the ER, and, in addition, P104 antagonized E_2-induced MCF-7BUS cell proliferation [252]. SM6Met, a DME of *C. subternata*, like P104, has been shown to bind to the ER with a lower affinity than E_2 [298] and transactivated an ERE-containing promoter reporter construct with a lower potency, but not efficacy, than that of E_2 [298]. SM6Met also induced MCF-7BUS cell proliferation with a lower potency than that of E_2 [298]. The water extract of *C. subternata*, cup-of-tea, has also been shown to induce MCF-7BUS cell proliferation with both a lower potency and efficacy than that of E_2 [298]. These findings warrant further investigation into how the phytoestrogenic extracts of *Cyclopia* would modulate the molecular targets involved in the prevention and treatment of breast cancer.

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Chapter 3

Cyclopia extracts act as ERα antagonists and ERβ agonists, *in vitro* and *in vivo*.

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PLoS ONE 2013; 8(11): e79223. doi:10.1371/journal.pone.0079223

With regard to Chapter 3, pp. 62-112, the nature and scope of my contribution were as follows:

Nature of contribution	**Extent of contribution (%)**
Conceived and designed experiments Performed experiments & Analysed data: • Figs. 1-8, Figs. S1-S5, • Table S1 Wrote paper Interpreted data Revised article critically for important intellectual content	• 100% of all Figures • 50% of all Tables

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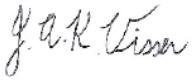
Signature of candidate: 
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Declaration by co-authors:
The undersigned hereby confirm that

1. the declaration above accurately reflects the nature and extent of the contributions of the candidate and the co-authors to Chapter 3, pp. 62-112,
2. no other authors contributed to Chapter 3, pp. 62-112, besides those specified above, and
3. potential conflicts of interest have been revealed to all interested parties and that the necessary arrangements have been made to use the material in Chapter 3, pp. 62-112, of this dissertation.

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3.1. Introduction

Hormone replacement therapy (HRT), estrogens alone or in combination with progestins, is traditionally prescribed to women undergoing menopausal transition to alleviate symptoms associated with menopause [1], such as hot flashes, night sweats, sleeping problems, vaginal dryness, and osteoporosis [2-4]. However, a number of side effects have been associated with the use of HRT, for example, an increased occurrence of breast cancer [5,6], vaginal bleeding [7], and heart disease or strokes [6,8]. These side effects have led to reluctance among concerned consumers to use HRT and instigated a search for new estrogen analogues with an improved risk profile. Furthermore, it would be of great value if these analogues should also display chemo-preventative properties in breast tissue [9,10].

Estrogens elicit their biological effects by binding to transcription factors called estrogen receptors (ERs) in the target organ/tissue (uterus, ovary, vagina, liver, bone, and breast) [11-13]. The ER exists as two subtypes, namely ERα and ERβ [14]. Current estrogens in HRT activate both subtypes of ER in all tissues [14-19]. This attribute is beneficial in bone [18,20,21] and for hot flashes [18,21], but detrimental in the breast [6,21,22] and uterus [21,23] as it increases the risk of tumourigenesis. In contrast, the selective estrogen receptor modulators (SERMs), although not ER subtype specific [24,25], act as agonists in certain tissues, such as bone [26-28], and as antagonists in others, such as breast [9,10,29]. Although, the well-known SERMs, raloxifene and tamoxifen [30], have been shown to decrease the risk of breast cancer [18,31,32] and increase bone mineral density [26-28,33], they have also been linked to an increased risk of venous thromboembolism and occurrence of hot flashes, and can stimulate endometrial growth [28,34-36]. SERMs are thus not considered as suitable alternatives for HRT.

Physiologically, while ERα is associated with the promotion of cell proliferation that contributes to the occurrence of breast and endometrial cancer, several studies have shown that ERβ inhibits ERα-dependent cell proliferation and could prevent cancer development [15,22,37-43]. 17β-estradiol (E_2) has similar binding affinities for the two ER subtypes [44], and the subtypes stimulate the

transcription of both common and distinct subsets of E_2 target genes [13,17,39,45]. However, in many cases the degree of activation via ERβ is lower [44], despite the high ligand independent transcriptional activity of this subtype [46,47]. In light of the above, it has been suggested that the development of ER subtype specific ligands may herald the arrival of a new generation of estrogen analogues that may present a novel treatment for post-menopausal symptoms, which in addition, may prevent or decrease the occurrence of breast cancer [44,48,49]. An ideal or "designer" estrogen analogue or selective estrogen receptor subtype modulator (SERSM) has been postulated that would have the following attributes: act as an ERα selective antagonist [50], down-regulate ERα protein levels [50,51], selectively activate ERβ transcriptional pathways [15,19,24,43], and display anti-inflammatory properties by inhibiting transcription of pro-inflammatory genes to prevent the occurrence of post-menopausal osteoporosis [15,52]. Current examples of subtype specific ligands are, methyl-piperidino-pyrazole (MPP) (ERα antagonist) [53,54], diarylpropionitrile (DPN) (ERβ agonist) [55], ERB-041 (ERβ agonist) [56,57], liquiritigenin (ERβ agonist) [19], isolated from the plant extract MF101 (ERβ agonist) [24]. Phytoestrogens have been referred to as natural SERMs and can be both estrogenic as well as antiestrogenic [58-60]. Furthermore, although evidence in the literature shows that phytoestrogens can bind to both ER subtypes, they generally have a higher affinity for the ERβ subtype [61-63] as well as a higher transcriptional potency and efficacy via ERβ [63]. Despite conflicting evidence regarding doses of phytoestrogens and breast cancer risk [64,65], generally, findings have pointed the search in the direction of phytoestrogens and focused attention on phytoestrogen rich food sources as a possible source of the ideal SERSM.

One such source may be *Cyclopia* (family: Fabaceae), an indigenous fynbos plant from the Western Cape province of South Africa [66,67]. Traditionally, the "fermented" (oxidized) form of *Cyclopia*, has been consumed as a fragrant, caffeine free honeybush tea beverage with the "unfermented" form being introduced to the commercial market more recently [63,67,68]. Studies that investigated the chemical composition of *Cyclopia* have shown that phenolic compounds with estrogenic

activity, for example luteolin, eriodictyol, naringenin, and formononetin, are present in various species of *Cyclopia* [63,68-72]. Furthermore, although dried methanol extracts (DMEs) from plant material of two species of *Cyclopia*, *C. genistoides* and *C. subternata*, have been shown to bind to the ERs and are able to transactivate an ERE-containing promoter reporter construct [62,63,68], only the extract from *C. genistoides* was investigated for ER subtype specificity and found to transactivate only through ERβ, despite binding to both subtypes [62,63]. In addition, studies by Verhoog *et al*. [63] and Mfenyana *et al*. [68] showed that although extracts of *Cyclopia* are able to induce proliferation of the ERα and ERβ positive MCF-7 BUS cells, they antagonise E_2 induced cell proliferation.

The current study was prompted by the findings of Verhoog *et al.* [62,63] that the *Cyclopia* extract, P104, although binding to both receptors and with a much higher affinity for ERα, was able to activate an ERE-containing promoter reporter construct only via ERβ. As the possibility of ERα antagonism by *Cyclopia* extracts had not been addressed in previous studies it appeared essential to evaluate ERα antagonism while also re-evaluating ERβ agonism. The combination of ERα antagonism and ERβ agonism may be especially relevant for the chemoprevention of breast cancer as ER antagonism serves as the basis of current chemo-preventative agents [29,31,32,73,74], while ERβ specific agonists have recently been identified as having potential for the chemoprevention of breast cancer [19,22]. In addition, this combination might be advantageous for the treatment of menopausal symptoms as an ERβ agonist has been shown to alleviate both hot flashes and the surge of inflammation related diseases during menopause [24,52], while an ERα antagonist would not result in hyperplasia of the uterus, commonly associated with ERα agonists [15,52]. Thus, in this study, we evaluate the potential of several extracts of *Cyclopia* to act as ERα antagonists and ERβ agonists and demonstrate that all extracts display ERα antagonism, while two also display ERβ agonism. In addition, all extracts antagonise E_2-induced MCF-7BUS cell proliferation, one extract displays anti-inflammatory activity, and the two tested extracts do not stimulate uterine growth. These results suggest that the *Cyclopia* extracts, which display ERα antagonism and ERβ agonism,

have positive attributes that could possibly be further exploited for the development of safer drugs for the treatment or prevention of osteoporosis or pre-menopausal symptoms.

3.2. Material and methods

3.2.1. Ethics statement

Animal care and experimental procedures were conducted with strict adherence to the accepted standards for the use of animals in research and teaching as reflected in the South African National Standards 10386: 2008. Stellenbosch University ethics committee approved this study (ethical approval reference: 11NB_LOU01).

3.2.2. Test Compounds

17β-Estradiol (E_2), genistein, luteolin, enterodiol, phorbol 12-myristate 13-acetate (PMA) and fulvestrant (ICI 182,780) were obtained from Sigma-Aldrich®, South Africa, and coumestrol was obtained from Fluka™ Analytical, Sigma-Aldrich®, South Africa. The *Cyclopia* extracts used for *in vitro* studies, P104 [62], SM6Met [68] and cup-of-tea [68], were previously prepared, while for *in vivo* studies new SM6Met and cup-of-tea extracts were prepared as previously described [68]. E_2, genistein, luteolin, enterodiol, coumestrol and *Cyclopia* extract stock solutions were prepared in dimethylsulfoxide (DMSO).

3.2.3. High-performance liquid chromatography (HPLC) analysis of C. subternata extracts

The newly prepared SM6Met and cup-of-tea extracts were analyzed using HPLC. Extracts and stock solutions of standards were prepared in DMSO and aliquots frozen at -20°C until needed for analysis. For experimental analysis ascorbic acid was added to defrosted standards and extracts to a final concentration of 9.8 mg/ml. The mixtures were then filtered using Millex-HV syringe filters (Millipore) with a 0.22 µm pore size.

Analyses were performed on an Agilent 1200 HPLC consisting of an in line degasser, diode-array detection (DAD), column oven, autosampler and quaternary pump, controlled by Chemstation software (Agilent Technologies, Santa Clara, CA). The HPLC method previously described by De

Beer *et al*. [75] was used to quantify the major phenolic compounds in *C. subternata* extracts: A Gemini-NX C18 (150 × 4.6 mm; 3 µm; 110 Å) column was used in conjunction with 2% acetic acid (A) and acetonitrile (B) as mobile phases. Injection volumes ranged from 10-20 µl for standards and 5-50 µl for the extracts. Separation was performed at a flow rate of 1 ml/min with the following mobile phase gradient: 0-2 min (8% B), 2-27 min (8-38% B), 27-28 min (38-50% B), 28-29 min (50% B), 29-30 min (50-8% B), 30-40 min (8% B); at a temperature of 30°C.

The dihyrochalcones, flavanones and benzophenones were quantified at 288 nm, whereas the xanthones, flavones and phenolic acids were quantified at 320 nm. A calibration curve consisting of seven points was set up for all the available standards (mangiferin (Sigma-Aldrich®, South Africa), isomangiferin (Chemos GmbH, Germany), luteolin (Extrasynthese, France), eriocitrin (Extrasynthese, France), hesperidin (Sigma-Aldrich®, South Africa), protocatechuic acid (Fluka™ Analytical, Sigma-Aldrich®, South Africa)) and also standards needed to calculate equivalent values (aspalathin (kind gift from Prof. Gelderblom, PROMEC unit, Medical Research Council, Tygerberg, South Africa), apigenin (Fluka™ Analytical, Sigma-Aldrich®, South Africa), and nothofagin (kind gift from Prof. Gelderblom, PROMEC unit, Medical Research Council, Tygerberg, South Africa)). Iriflophenone-3-*C*-*β*-glucoside and iriflophenone-di-*O*,*C*-hexoside was quantified using iriflophenone-3-*C*-glucoside isolated from *C. genistoides* (personal communication from Dr. D. de Beer). Scolymoside and vicenin-2 were expressed as luteolin and apigenin equivalents, respectively, as no authentic reference standards were available for these compounds. Also phloretin-3',5'-di-*C*-glucoside and 3-hydroxyphloretin-3',5'-di-*C*-hexoside were expressed in terms of nothofagin (3-hydroxyphloretin-3'-*C*-glucoside) and aspalathin (3-hydroxyphloretin-5'-*C*-glucoside) equivalents, respectively.

3.2.4. Cell Culture

COS-1, African green monkey kidney fibroblast cells (ATCC, United States of America), and MCF-7BUS human breast cancer cells [76] (a kind gift from A. Soto, Tufts University, Boston, Massachusetts, United States of America) were maintained in high glucose (4.5 g/L) Dulbecco's

modified eagle's medium (DMEM) (Sigma-Aldrich®) supplemented with 10% FCS (Highveld Biologicals, South Africa), 100 IU/ml penicillin and 100 µg/ml streptomycin (Gibco, Invitrogen™, South Africa), 2mM glutamine (Merck), 44mM sodium-bicarbonate (Gibco), 1mM sodiumpyruvate (Gibco, Invitrogen Corporation), and 0.1mM non-essential amino acids (Gibco). All cells were maintained in a humidified cell incubator, set at 97% relative humidity and 5% CO_2 at 37˚C. For the cell proliferation assays (MTT assay) MCF-7BUS cells were withdrawn from 100 IU/ml penicillin and 100µg/ml streptomycin for seven days prior to use.

3.2.5. MTT assay

On day one MCF-7BUS cells were seeded into 96-well tissue culture plates at a concentration of 2500 cells/well and allowed 24 hours to settle. The next day cells were washed with 200 µl/well pre-warmed PBS and the medium was changed to DMEM without phenol red supplemented with 5% charcoal treated FCS (Highveld Biologicals, South Africa) and incubated for 24 hours. After incubation the cells were treated for 48 hours with increasing concentrations test compounds and *Cyclopia* extracts in the presence or absence of 10^{-9}M E_2 where after the colorimetric MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay, adapted from Verhoog *et al.* [63] and Mfenyana *et al.* [68], was performed. Briefly, the MTT assay entails that 4 hours before the end of the incubation period the assay medium is changed to 150 µl DMEM without phenol red, but supplemented with 5% charcoal stripped FCS, and 50 µL of MTT (methylthiazolyldiphenyl-tetrazolium) (Sigma-Aldrich®) solution (5 mg/ml) is added to each well. Cells are then incubated for four hours at 37°C, the medium removed, and 200 µL of solubilisation solution (DMSO) added to each well. The plate is then covered with foil, shaken at room temperature for 5 min, and the absorbance read at 550 nm on a BioTek® PowerWave 340 spectrophotometer. All assays included a negative solvent control, which consisted of 0.1% (v/v) DMSO only. Results are expressed as fold induction relative to solvent.

3.2.6. Promoter reporter studies

MCF-7BUS and COS-1 cells were seeded in sterile 10 cm tissue culture plates at a concentration of 2 x 10^6 cells/plate and allowed 24 hours to settle. On day two the cells were rinsed once with sterile phosphate buffered saline (PBS) (pre-warmed to 37˚C), medium changed to DMEM without phenol red supplemented with 10% charcoal treated FCS and 1% penicillin and streptomycin mixture, and cells were transfected.

3.2.6.1. Plasmids

Human (h) ERα (pSG5-hERα [77]) and ERβ (pSG5-hERβ [78]) expression plasmids were kind gifts from F. Gannon (European Molecular Biology Laboratory, Heidelberg, Germany). The ERE-containing promoter reporter construct (ERE.vit2.luc) was a kind gift from K. Korach, National Institute of Environmental Health Science, U.S. [79] and the NFκB-containing promoter reporter construct (p(IL6κB)350hu.IL6Pluc+ [80]) was a kind gift from G. Haegeman, University of Ghent, Ghent, Belgium. pGL2-Basic (Promega Corporation, Madison, Wisconsin, U.S.A.) was used as an empty vector.

3.2.6.2. Transactivation

To test transactivation through ERα COS-1 cells were transfected with 150 ng hERα and 6000 ng of an ERE-containing promoter reporter construct. To test transactivation through ERβ COS-1 cells were transfected with 150 ng hERβ, 3000 ng of an ERE-containing promoter reporter construct, and 3000 ng empty vector. MCF-7 BUS cells (which contain endogenous hERα and hERβ) were transfected with 3000 ng of an ERE-containing promoter reporter construct and 3000 ng empty vector. The amount of promoter reporter construct for each test model that was selected was determined by the highest E_2 induction achieved (Figure S1).

3.2.6.3. Transrepression

To test transrepression through ERα COS-1 cells were transfected with 150 ng hERα, 1500 ng of an NFκB-containing promoter reporter construct and 4500 ng empty vector. To test transrepression through ERβ COS-1 cells were transfected with 150 ng hERβ, 4500 ng of an NFκB-containing

promoter reporter construct and 1500 ng empty vector. MCF-7BUS cells (which contain endogenous hERα and hERβ) were transfected with 6000 ng of an NFκB-containing promoter reporter construct. The amount of promoter reporter construct for each test model that was selected was determined by the most effective E_2 repression of PMA induction achieved (Figure. S2)

All transfections were performed using FuGENE^{TM} 6 transfection reagent (Roche Applied Science, South Africa) as described by the manufacturer. Cells were left for 24 hours, replated in sterile 24-well tissue culture plates at a concentration of 5 x 10^4 cells/well and allowed 24 hours to settle. Cells were treated for 24 hours with test compounds and *Cyclopia* extracts and lysed overnight with 50 µl lysis buffer [0.2% (vol/vol) Triton, 10% (vol/vol) glycerol, 2.8% (vol/vol) Tris-phosphate-EDTA, and 1.44 mM EDTA] per well at -20 ˚C. Luciferase activity was determined using the luciferase assay kit (Promega Corporation, Anatech, South Africa) according to the manufacturer's instructions and normalized for protein content (Bradford assay [81]). Results are expressed as fold induction relative to solvent.

3.2.7. Western Blot

Cell lysates from COS-1 cells transfected with either ERα (150 ng hERα/10 cm plate) or ERβ (150 ng hERβ/10 cm plate) and MCF-7BUS cells were prepared by adding lysis buffer A (10mM Hepes pH 7.5 (Gibco, Invitrogen Corporation), 1.5mM $MgCl_2$, 10mM KCl, 0.1% NP-40 (Roche Applied Science) and Complete Mini protease inhibitor cocktail (Roche Applied Science), shaking on ice for 15 min and frozen overnight at -20˚C.

On thawing, lysate were transferred to 1.5ml Eppendorf tubes on ice, centrifuged for 10 min at 12 000 x g at 4˚C and the cleared lysate was transferred to 1.5ml Eppendorf tubes on ice, alliquoted and stored at -20˚C until assayed. Lysates (20µl) were separated on a 10% SDS-PAGE gel. Following electrophoresis, proteins were electro-blotted and transferred to a Hybond-ECL nitrocellulose membrane (Amersham Biosciences, South Africa), which was probed for ERα (diluted 1:500), ERβ (1:250) and GAPDH (1:500). Proteins were visualized using HRP labeled anti-rabbit antibody for ERα (1:2500) and ERβ (1:1000), or HRP labeled anti-mouse antibody for

GAPDH (1:5000), and ECL Western blotting detection reagents (Pierce®, Thermo Fisher Scientific Inc., U.S.A.) and medical x-ray film (Axim (PTY) LTD., South Africa). All antibodies, primary [ERα (HC-20), cat# sc-543, ERβ (H-150), cat# sc-8974, and GAPDH (0411), cat# sc-47724] and secondary (anti-rabbit, cat# sc-2005, and anti-mouse, cat# sc-2030), were purchased from Santa Cruz Biotechnology, Inc., U.S.A.

3.2.8. Animal care

Immature female Wistar rats were obtained from the Stellenbosch University, South Africa, breeding unit and were received as weanlings on postnatal day 18. The animals had free access to standard rat feed (Pure Harvest Rat Feed, Afresh Vention (PTY) Ltd, South Africa) and drinking water. The animals were housed in a 12 hour light-dark cycle at a constant temperature of 20 °C in EHRET individually ventilated cages (EHRET, Emmedingen, Germany). The animals were allowed at least 24 hours to acclimatize before the onset of experimental procedures.

3.2.9. Immature rat uterotrophic assay

The immature rat uterotrophic assay was performed according to methods previously described by Kanno *et al.* [82] and de Lima *et al.* [83]. Immature Wistar rats (21 days) were randomly assigned to groups (n=10) and treated daily with E_2, genistein, *Cyclopia* extracts, or vehicle control (sterile PBS) by oral gavage for three consecutive days. The dose volume was 200 µl/day. The test compounds were suspended in sterile PBS and the solution was kept homogenous by stirring before administration. General health, vaginal opening, and body weight was monitored daily and recorded. On day four, approximately 24 hours after last dose, animals were weighed and sacrificed by administration of a high dose of Isoflurane (2-chloro-2-(difluoromethoxy)-1,1 1-trifluoro-ethane), (Safeline pharamceuticals Pty (Ltd)). Livers were removed and weighed. Uteri were removed, cleaned of excess fat, photographed, weighed, pierced to remove luminal fluids, and blotted uterine weights were obtained immediately.

3.2.10. Evaluation/Monitoring of vaginal opening of Wistar rats for extended period

Immature Wistar rats (21 days) were randomly assigned to groups (n=10) and treated daily with E_2, *Cyclopia* extracts, or vehicle control (sterile PBS) by oral gavage for 30 consecutive days. The dose volume had to be increased gradually from 200 µl/day to 400 µl/day as animals increased in body weight. The test compounds were suspended in sterile PBS and the solution was kept homogenous by stirring before administration. General health, vaginal opening, and body weight was monitored daily and recorded. On day 30 animals were weighed and sacrificed by administration of a high dose of Isoflurane.

3.2.11. Data manipulation and statistical analysis

The GraphPad Prism® version 5.10 for Windows (GraphPad Software) was used for graphical representations and statistical analysis. One-way ANOVA and Dunnett's post-test comparing all columns to the solvent control were used for statistical analysis and significance is displayed on the graphs. For all experiments the error bars represent the SEM of at least two independent experimental repeats, except for *in vivo* studies where the error bars represent the SEM of the number of animals used.

3.3. Results

3.3.1. HPLC analyses of extracts of Cyclopia

New SM6Met and cup-of-tea extracts were prepared from the same harvesting of *C. subternata* previously used to prepare these extracts [68]. HPLC analysis was performed on these newly prepared SM6Met and cup-of-tea extracts (Table 1). Prior HPLC results of previously prepared P104 [63] and SM6Met [68] extracts are also shown in Table 1. The results indicate the presence of the xanthones, mangiferin and isomangiferin, the flavones, scolymoside, luteolin, and vicenin-2, the flavanones, eriocitin and hesperidin, the dihydrochalcones, phloretin-3,5-di-*C*-glucoside and aspalathin, the benzophenones, iriflophenone-3-*C*-β-glucoside and iriflophenone-di-*O*,*C*-hexoside, and the phenolic carboxylic acid, protocatechuic acid. P104, a DME from *C. genistoides*, contained

more mangiferin and isomangiferin than SM6Met, a DME from *C. subternata*, while, the cup-of-tea extract from the same species contained the least.

Table 1. Major polyphenols present in previously and newly prepared extracts of *Cyclopia* as determined by HPLC.

	Extract			
	Previously prepared		**Newly prepared**	
Polyphenol [% of dry extract (g/100g dry extract)]	**P104 [63]** ***C.genistoides***	**SM6Met [68]** ***C.subternata***	**SM6Met** ***C.subternata***	**Cup-of-tea** ***C.subternata***
Mangiferin	3.606	1.850	1.899	1.000
Isomangiferin	5.094	0.750	0.645	0.420
Luteolin	0.096	0.040	0.040	0.018
Scolymoside (luteolin-7-*O*-rutinoside)	nt[a]	1.820[c]	1.289	0.876
Vicenin-2 (apigenin-6,8-di-*C*-glucoside)	nt	nt	0.089	0.065
Eriocitrin (eriodictyol-7-*O*-rutinoside)	nd[b]	1.250	0.846	0.600
Hesperidin (hesperitin-7-*O*-rutinoside)	nt	1.870	2.049	0.935
Phloretin-3',5'-di-*C*-glucoside	nt	1.270[d]	1.278	0.939
3-Hydroxyphloretin-3',5'-di-*C*-hexoside	nt	nt	0.700	0.582
Iriflophenone-3-*C*-β-glucoside	nt	nt	0.669	0.590
Iriflophenone-di-*O*,*C*-hexoside	nt	nt	0.958	0.896
Protocatechuic acid	nt	nt	0.113	0.082

[a]Not tested
[b]Not detected
[c]Previously "Unknown 1" was quantified as luteolin equivalents as it appeared to be a flavone.
[d]Previously "Unknown 2" was quantified as hesperidin equivalents as it appeared to be a flavanone.

Luteolin was present in all of the extracts, albeit at small amounts, with the P104 extract containing the largest amount, followed by the SM6Met extracts, and with the cup-of-tea extract containing the least. The luteolin rutinoside, scolymoside, was not evaluated in P104. The DMEs of *C. subternata* contained more scolymoside, eriocitrin, hesperidin, and phloretin-3,5-di-*C*-glucoside than the cup-of-tea extract. The newly prepared DME, SM6Met, contained higher amounts than the cup-of-tea extract of compounds not previously tested for, namely, iriflophenone-3-*C*-β-glucoside, iriflophenone-di-*O*,*C*-hexoside, 3-hydroxyphloretin-3',5'-di-*C*-hexoside, vicenin-2, and protocatechuic acid. In general the DMEs contained higher concentrations of the polyphenols quantified (Table 1) than the water extract.

3.3.2. Methanol extracts of Cyclopia act as agonists of ERβ, while all extracts antagonize E_2-induced activation via ERα.

To evaluate ERα antagonism while also re-evaluating ERβ agonism COS-1 cells were transiently transfected with either ERα (Figs. 1 A, C) or ERβ (Figs. 1 B, D) and an ERE-containing promoter reporter construct. Agonism was tested in the absence (Figs. 1 A, B) and antagonism in the presence (Figs. 1 C, D) of 10^{-9} M E_2. Three *Cyclopia* extracts, from two species, *C. genistoides* and *C. subternata*, were tested. Two were methanol extracts, P104 and SM6Met, and one was a water extract, cup-of-tea. In addition we investigated an example from each of the major classes of phytoestrogens: genistein, a well-studied isoflavone, enterodiol, a lignin, and coumestrol, a coumestan [84,85]. Luteolin, an estrogenic polyphenol [71], was also included as it was found be present in all of the *Cyclopia* extracts (Table 1), while E_2 represents the major endogenous estrogen [86,87].

E_2 induced ERα mediated transactivation in a dose dependent manner with significant induction at two concentrations of E_2, 10^{-9} M (2.7×10^{-4} µg/ml) (2.5 ± 0.5 fold) and 9.8 µg/ml (3.6×10^{-5} M) (3.9 ± 0.7 fold), but not at the lowest concentration of 10^{-11} M (2.7×10^{-6} µg/ml) (Fig. 1A). The same trend was seen for ERβ (2.5 ± 0.5 fold at 10^{-9} M and 2.7 ± 0.4 fold at 9.8µg/ml) (Fig. 1B), although at the highest concentration of E_2 higher induction was observed via ERα than via ERβ (3.9 ± 0.7 vs. 2.7 ± 0.4 fold). Although the 9.8 µg/ml E_2 represents a supra-physiological concentration the 10^{-11} M and 10^{-9} M E_2 concentrations reflect the pre- and post-menopausal levels of E_2 respectively [88]. At the concentration of 9.8 µg/ml, genistein (3.6×10^{-5} M), luteolin (3.4×10^{-5} M), and coumestrol (3.7×10^{-5} M) significantly activated gene transcription through both of the ER subtypes (Figs. 1A, B). Enterodiol, however, could not significantly activate gene transcription through either of the subtypes at the concentration of 9.8 µg/ml (3.2×10^{-5} M) (Figs. 1A, B). None of the *Cyclopia* extracts were able to induce activation through ERα (Fig. 1A), but both the methanol extracts, P104 and SM6Met, were able to significantly activate transcription through ERβ (2.4 ± 0.4 and 2.5 ± 0.3 fold, respectively).

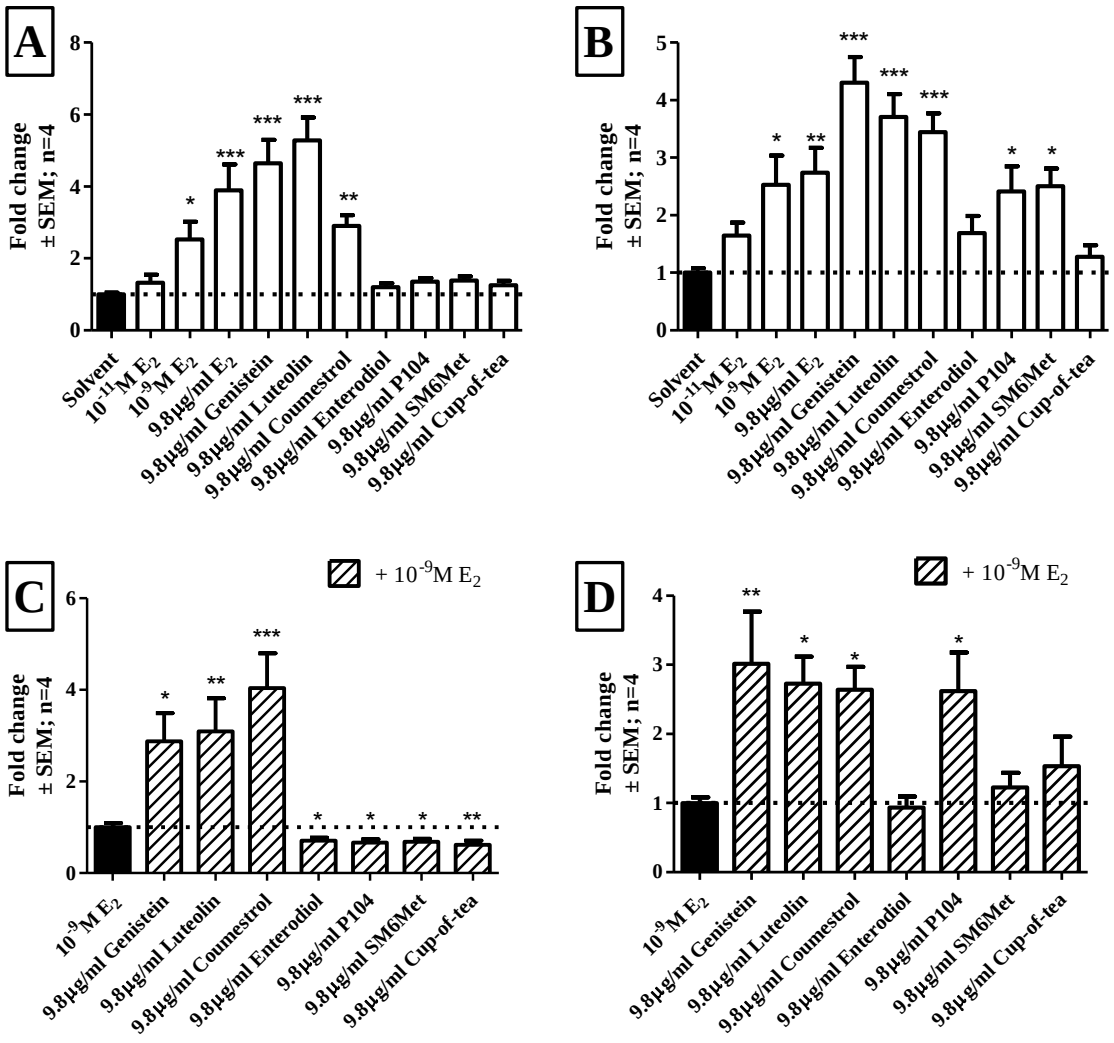


Figure 1. Evaluation of ER subtype specific agonism and antagonism of transactivation of an ERE-containing promoter reporter construct in COS-1 cells. COS-1 cells were transfected with either (A and C) pSG5-hERα or (B and D) pSG5-hERβ and ERE.vit2.luc and treated for 24 hours with a series of test compounds or extracts. To test agonism cells were treated with test compounds or extracts alone, (A and B), while, to test for antagonism cells were treated with test compounds or extracts in the presence of 10^{-9}M E_2 (C and D). Statistical analysis was done using One-way ANOVA with Dunnett's post-test comparing all columns to the solvent control (*, P<0.05; **, P<0.01; ***, P<0.001). The dotted line through the bars represents the values for solvent control. Mean ± SEM is of four independent experiments done in triplicate.

To address antagonism, transactivation in the presence of 10^{-9} M E_2 was evaluated (Fig. 1C and D). The phenolic compounds, genistein, luteolin, and coumestrol were not antagonists but had an additive effect on E_2-induced activation via both receptor subtypes (Figs. 1C and D), confirming their agonism through both subtypes (Fig. 1A and B). Enterodiol in contrast, however, only displays ERα antagonism (0.7 ± 0.1 fold vs. E_2 activation set as 1) (Fig. 1C). All of the *Cyclopia* extracts significantly antagonized ERα mediated E_2-induction (P104, 0.7 ± 0.1, SM6Met, 0.7 ± 0.1,

and cup-of-tea, 0.6 ± 0.1 fold), however, only P104 had an additive effect on the E_2-induced activation through ERβ (Fig. 1D). In conclusion, the methanol extracts of *Cyclopia* are ERβ agonists and all extracts are ERα antagonists.

3.3.3. In MCF-7BUS cells expressing both ER subtypes all extracts of Cyclopia transactivate an ERE-driven promoter reporter construct.

Most tissues affected by menopause and/or implicated in HRT side effects, such as uterus, bone, and breast, contain both ER subtypes [89]. Thus, having shown that methanol extracts of *Cyclopia* are ERβ agonists and all extracts are ERα antagonists in a system where the ER subtypes were evaluated separately, we were interested in investigating the transactivation potential of *Cyclopia* extracts in a system where both subtypes are present.

MCF-7BUS cells, containing both ERα and ERβ (Fig. 2A), were transfected with an ERE-containing promoter reporter construct and both agonism (Fig. 2B) and antagonism (Fig. 2C) were tested. Although strong transactivation was seen with E_2, none of the polyphenols on their own were able to significantly activate gene transcription in this system where both ER subtypes are present (Fig. 2B), despite the fact that these polyphenols transactivate when the ER subtypes function in isolation (Fig. 1A and B). Furthermore, most of the polyphenols, excluding coumestrol, antagonized E_2 induction when both ER subtypes are together (Fig. 2C), whereas when the subtypes were expressed separately only enterodiol showed ERα antagonism (Fig. 1C). In contrast to the polyphenols, the extracts of *Cyclopia*, P104 (3.4 ± 0.5 fold), cup-of-tea (3.4 ± 0.5 fold) and, SM6Met (3.5 ± 0.6 fold), were able to activate transcription to a similar extent as 10^{-9} M E_2 (3.8 ± 0.3 fold) (Fig. 2B). These results, together with the fact that the *Cyclopia* extracts did not antagonize E_2 induction (Fig. 2C), suggests that when both ER subtypes are co-expressed the *Cyclopia* extracts act as agonists, whereas when the ER subtypes are expressed separately they only act as agonists through ERβ and antagonize ERα induction.

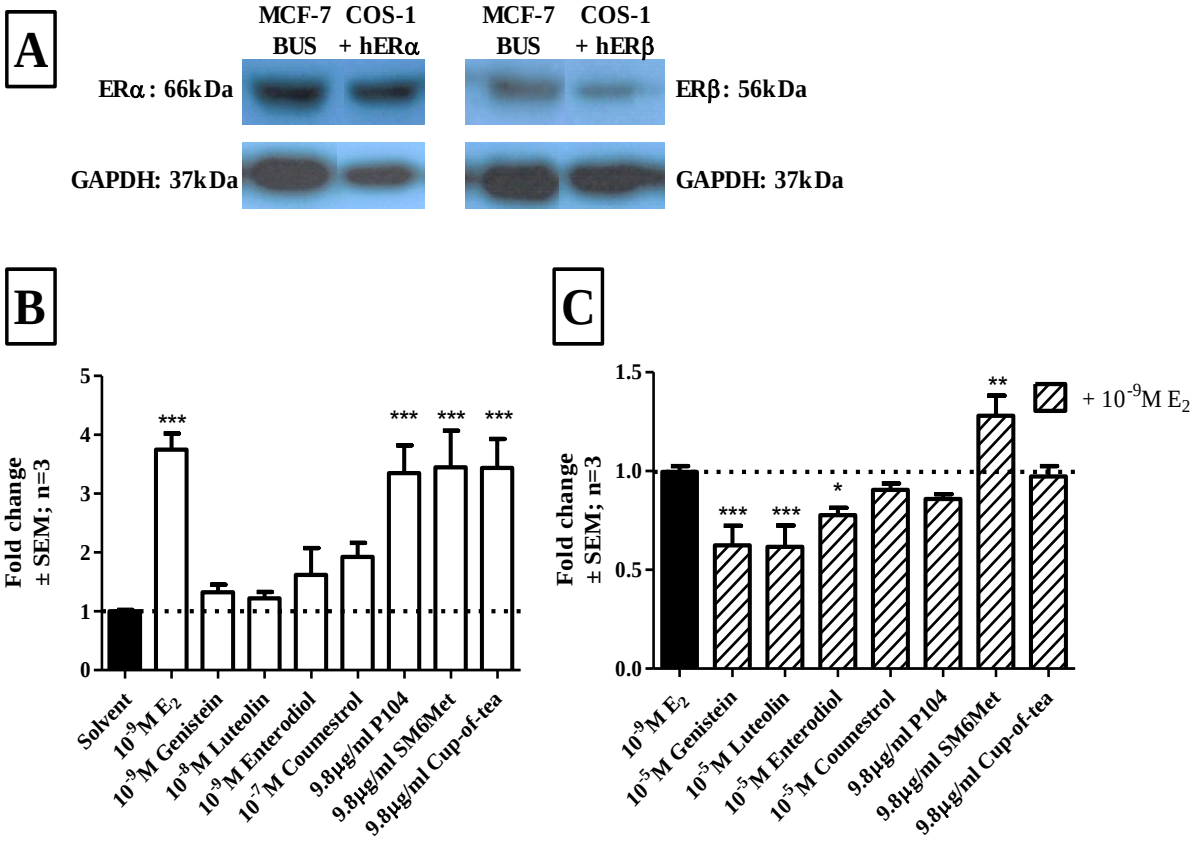


Figure 2. Evaluation of transactivation of an ERE-containing promoter reporter construct in MCF-7BUS cells expressing both ERα and ERβ. MCF-7BUS cells, with endogenous ERα and ERβ (A), were transfected with ERE.vit2.luc and treated for 24 hours with a series of test compounds or extracts. To test agonism cells were treated with test compounds or extracts alone (B), while, to test for antagonism cells were treated with test compounds or extracts in the presence of 10^{-9}M E_2 (C). Statistical analysis was done using One-way ANOVA with Dunnett's post-test comparing all columns to the solvent control (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$). The dotted line through the bars represents the values for solvent control. Mean ± SEM is of three independent experiments done in triplicate.

3.3.4. An extract of C. genistoides represses NFκB activation via ERα and ERβ whereas the extracts of C. subternata are ERβ antagonists.

The decline in estrogen levels during menopause leads to a surge in the occurrence of inflammatory disorders [52,90-92]. Furthermore, NFκB, a pro-inflammatory transcription factor, is involved in the development of breast cancer [93-95]. Taking this into account we wanted to evaluate the ability of *Cyclopia* extracts to repress the activation of an NFκB-containing promoter reporter construct by transfecting COS-1 cells with said construct and either ERα (Figs. 3A, C, E) or ERβ (Figs. 3B, D, F).

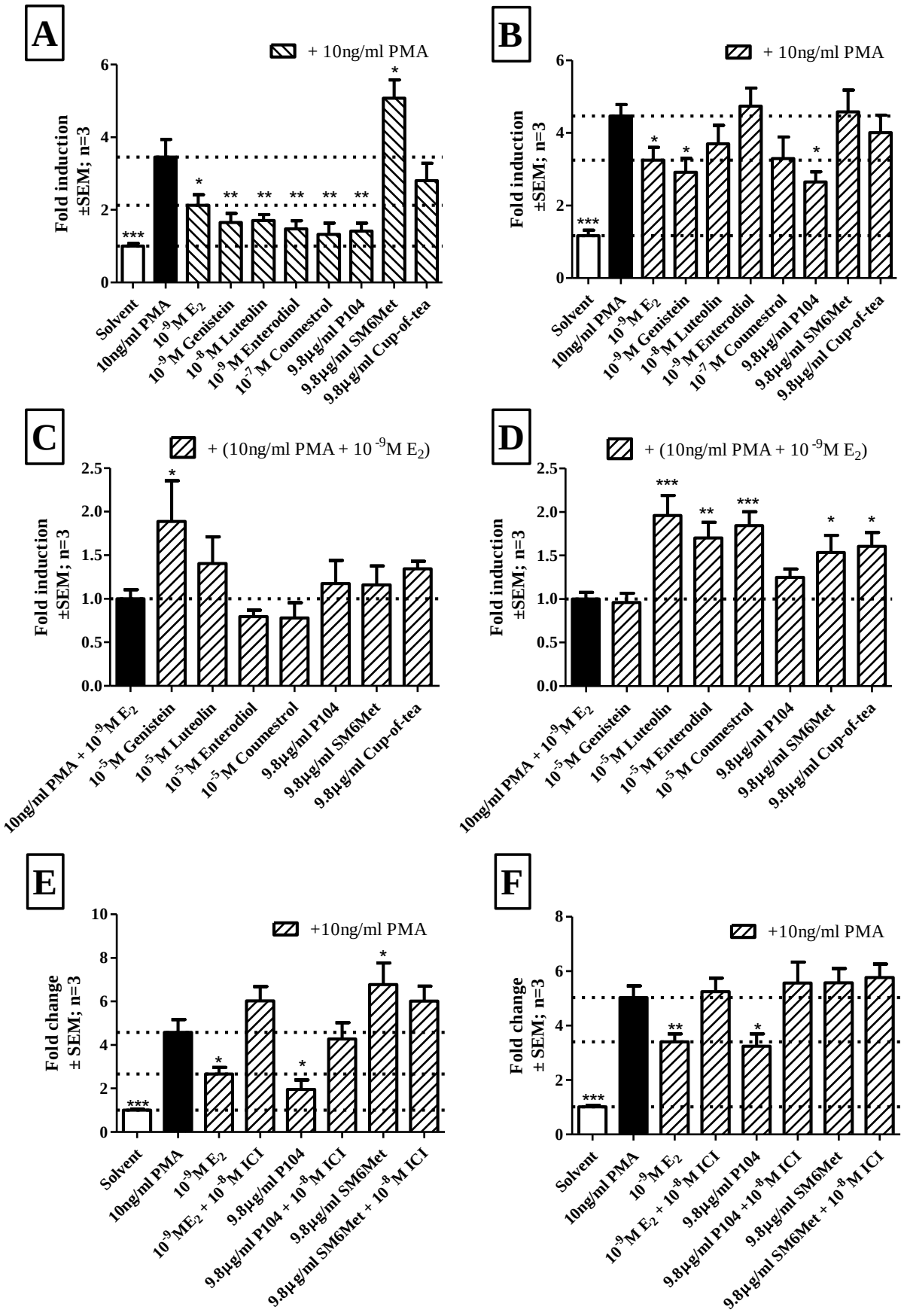


Figure 3. Evaluation of ER subtype specific agonism and antagonism of an NFκB-containing promoter reporter construct in COS-1 cells. COS-1 cells were transfected with either (A, C, and E) pSG5-hERα or (B, D, and F) pSG5-hERβ and p(IL6kB)350hu.IL6Pluc+ and treated for 24 hours with a series of test compounds or extracts. To test agonism cells were treated with test compounds or extracts alone, (A and B), while, to test antagonism cells were treated with test compounds or extracts in the presence of 10^{-9} M E_2 (C and D). To ascribe the observed effect to the ER we treated cells with P104 and SM6Met in the absence or presence of the ER antagonist ICI 182,870 (E and F). Statistical analysis was done using One-way ANOVA with Dunnett's post-test comparing all columns to either (A, B, E, and F) 10ng/ml PMA or (C and D) 10ng/ml PMA + 10^{-9}M E_2 (*, P<0.05; **, P<0.01; ***, P<0.001). The dotted lines through the bars represent the values for either (A, B, E, and F) solvent control, 10ng/ml PMA, or 10ng/ml PMA + 10^{-9}M E_2 or (C and D) 10ng/ml PMA + 10^{-9}M E_2. Mean ± SEM is of three independent experiments done in triplicate.

In addition, this system would provide information concerning the behavior of *Cyclopia* extracts in a transrepression model. Agonism was tested in the absence (Figs. 3A, B) and antagonism (Figs. 3C, D) in the presence of 10^{-9} M E_2.

PMA (phorbol 12-myristate 13-acetate, an activator of NFκB driven gene expression [96,97]) activation of the NFκB-containing construct was repressed by E_2 via both receptor subtypes (Fig. 3A and B) with a more pronounced repression through ERα (38.6% vs. 27.2%). Like E_2, all of the polyphenols, as well as P104 (*C. genistoides* extract), acted as ERα agonists by repressing PMA activation (genistein 52.1%, luteolin 50.6%, enterodiol 57.4%, coumestrol 61.8%, and P104 59.2%) (Fig. 3A). Furthermore, genistein (34.8% repression) and P104 (40.7% repression), like E_2, also displayed significant ERβ agonism (Fig. 3B). Therefore, in our transrepression model P104 is not an ERβ selective agonist, but displays agonism via both subtypes. The water extract of *C. subternata*, cup-of-tea, was unable to repress PMA induction through either ERα or ERβ (Figs 3A, B) while the methanol extract, SM6Met, also unable to repress PMA induction through either subtype, significantly added to the activation observed with PMA alone via ERα (5.1 ± 0.5 vs. 3.5 ± 0.5) (Fig. 3A, B).

Antagonism was evaluated in the presence of 10^{-9} M E_2 and only genistein (Fig. 3C) had a significant effect via ERα by antagonizing E_2 repression of PMA activation. The polyphenols, luteolin, enterodiol, and coumestrol, but not genistein, however, antagonized E_2 repression of PMA

activation via ERβ (Fig. 3D). Although none of the extracts displayed significant antagonism of ERα, the extracts of *C. subternata* displayed ERβ antagonism (Fig. 3D).

The result for SM6Met in Fig. 3A prompted us to investigate whether this effect was via ERα or if SM6Met is able to activate the NFκB-containing construct through another mechanism of action. Therefore, we repeated the experiment, for both receptor subtypes, with SM6Met, as well as P104, in the presence and absence of an ER antagonist, ICI 182,780 (Figs. 3E, F). The observed repression of PMA activation by E_2 and P104 via ERα and ERβ is abolished by ICI (Fig. 3E, F) and thus, the observed repression is indeed via the ER. SM6Met, like ICI, increases PMA activation through ERα (Fig. 3E) and both have no significant effect on PMA activation via ERβ (Fig. 3F). Furthermore, the increased transactivation observed with SM6Met in Fig. 3A may be attributed to residual E_2 remaining after stripping of FCS, as suggested by others [22], which would further support the contention that SM6Met is behaving as an ERα antagonist. In conclusion then the results suggest that for our transrepression model the methanol extract of *C. genistoides* (P104) is behaving like an ERα and ERβ agonist, while the methanol extract of *C. subternata* (SM6Met) is an ERα antagonist in the absence of E_2, and an ERβ antagonist in the presence of E_2.

3.3.5. In MCF-7BUS cells expressing both ER subtypes all extracts are agonists, while the water extract of C. subternata also displays antagonistic activity.

As we have shown that P104 is an ER agonist and SM6Met is an ER antagonist in a transrepression model where the ER subtypes function in isolation (Fig. 3), we wanted to test the effect of these extracts in a model where both subtypes are present as most tissues affected by menopause and/or implicated in HRT side effects contain both subtypes.

MCF-7BUS cells were transfected with an NFκB-containing promoter reporter construct and both agonism (Fig. 4A) and antagonism (Fig.4 B) evaluated. Strong repression was observed with E_2, the polyphenols, and P104 when both subtypes are present (Fig. 4A), which correlates with what was observed previously for ERα alone (Fig. 3A). However, for ERβ alone (Fig. 3B), significant repression was previously seen only with E_2, genistein, and P104 but not with luteolin, enterodiol,

and coumestrol. Unlike previous results, SM6Met behaved differently when subtypes were co-expressed than when the subtypes were expressed separately.

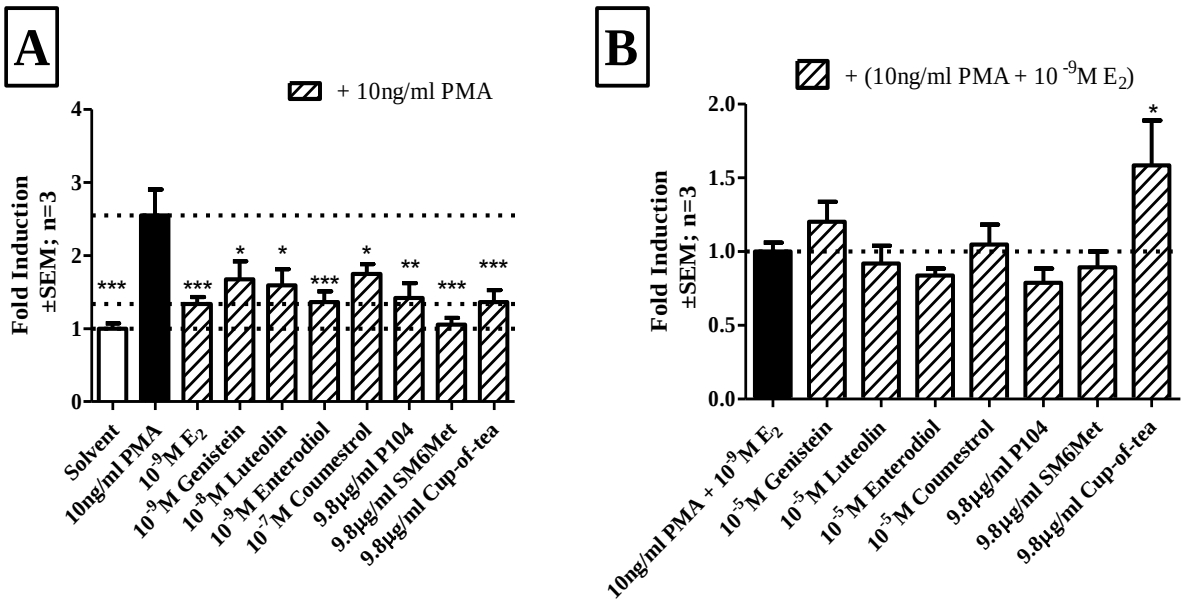


Figure 4. Evaluation of transrepression of an NFκB-containing promoter reporter construct in MCF-7BUS cells expressing both ERα and ERβ. MCF-7BUS cells were transfected with p(IL6kB)350hu.IL6Pluc+ and treated for 24 hours with a series of test compounds or extracts. To test agonism cells were treated with test compounds or extracts alone, (A), while, to test for antagonism cells were treated with test compounds or extracts in the presence of 10^{-9}M E_2 (B). Statistical analysis was done using One-way ANOVA with Dunnett's post-test comparing all columns to either (A) 10ng/ml PMA or (B) 10ng/ml PMA + 10^{-9}M E_2 (*, P<0.05; **, P<0.01; ***, P<0.001). The dotted lines through the bars represent the values for either (A) solvent control, 10ng/ml PMA, or 10ng/ml PMA + 10^{-9}M E_2 or (B) 10ng/ml PMA + 10^{-9}M E_2. Mean ± SEM is of three independent experiments done in triplicate.

It displayed agonism when subtypes are expressed together (Fig. 4A) while displaying antagonism when expressed separately (Fig. 3A and D). Similarly, where no agonist activity via either subtype alone was observed previously, the cup-of-tea extract was able to change its behavior when both subtypes are present by displaying ER agonism. Furthermore, antagonism in the presence of both subtypes was only seen with the cup-of-tea extract (Fig. 4B), while the subtype specific antagonism of genistein, luteolin, enterodiol, coumestrol, and SM6Met (Figs. 3C, D) is abrogated in the presence of both subtypes. Taken together, in a transrepression model, the DME of *C. genistoides*, P104, is an ER agonist in all models (Figs. 3A, B, and 4A), the DME of *C. subternata*, SM6Met, is an ERβ antagonist in the presence of E_2 (Fig. 3D), an ERα antagonist in the absence of E_2 (Fig. 3A, E), and an agonist in the presence of both ER subtypes (Fig. 4A), while the water extract of *C.*

subternata, cup-of-tea, is an ERβ antagonist (Fig. 3D) and an ER agonist/antagonist (Figs. 4A, B) in the presence of both subtypes. This differential behavior of the *Cyclopia* extracts in the transrepression model contrasts to similar behavior by the extracts in the transactivation model where all extracts displayed antagonism through ERα (Fig. 1) alone, while displaying agonism to ERβ (Fig. 1) alone or when both subtypes are expressed (Fig. 2).

3.3.6. Cyclopia extracts weakly induce proliferation of breast cancer cells but antagonizes E_2-induced breast cancer cell proliferation.

Having shown that *Cyclopia* extracts can modulate both transactivation and transrepression in the presence of both ER subtypes and when the subtypes are expressed alone, we wanted to re-evaluate agonism of P104 [63], SM6Met and cup-of-tea [68] (Fig. 5) and antagonism of P104 [63] (Fig. 6) and for the first time evaluate antagonism of SM6Met and cup-of-tea (Fig. 6) on MCF-7BUS breast cancer cell proliferation. Cell proliferation in MCF-7BUS cells constitutes an integrated model where not only the ER subtypes are co-expressed, but both transactivation and transrepression of endogenous genes contribute towards the final phenotype, whether it is proliferative or anti-proliferative [39,98-100].

The MTT cell proliferation assay using MCF-7BUS cells was used to address agonism (Fig.5A-H). Estrogen induced cell proliferation at a wide range of concentrations (10^{-6} M to 10^{-10} M) with the highest efficacy (2.1 ± 0.1 fold) observed at 10^{-9} M E_2 (2.7 x 10^{-4} µg/ml) (Fig. 5A). Like E_2, all of the polyphenols were also able to induce cell proliferation, but not to the same extent as E_2, with a maximum efficacy of: genistein, 1.5 ± 0.1 fold at 10^{-9} M (2.7 x 10^{-4} µg/ml) (Fig. 5B), luteolin, 1.5 ± 0.1 fold at 10^{-5} M (2.7 µg/ml) (Fig. 5C), coumestrol, 1.6 ± 0.1 fold at 10^{-6} M (3.0 x 10^{-1} µg/ml) (Fig. 5D), and enterodiol, 1.3 ± 0.1 fold at 10^{-9} M (3.0 x 10^{-4} µg/ml) (Fig. 5E). Similarly, all three extracts of *Cyclopia* induced proliferation of cells with a lower efficacy than E_2 with maximum efficacies of 1.5 ± 0.2 (significantly different from E_2), 1.3 ± 0.03 (significantly different from E_2), and 1.7 ± 0.2 (not significantly different from E_2) fold for 9.8 µg/ml of P104, cup-of-tea and SM6Met, respectively (Figs. 5F-H).

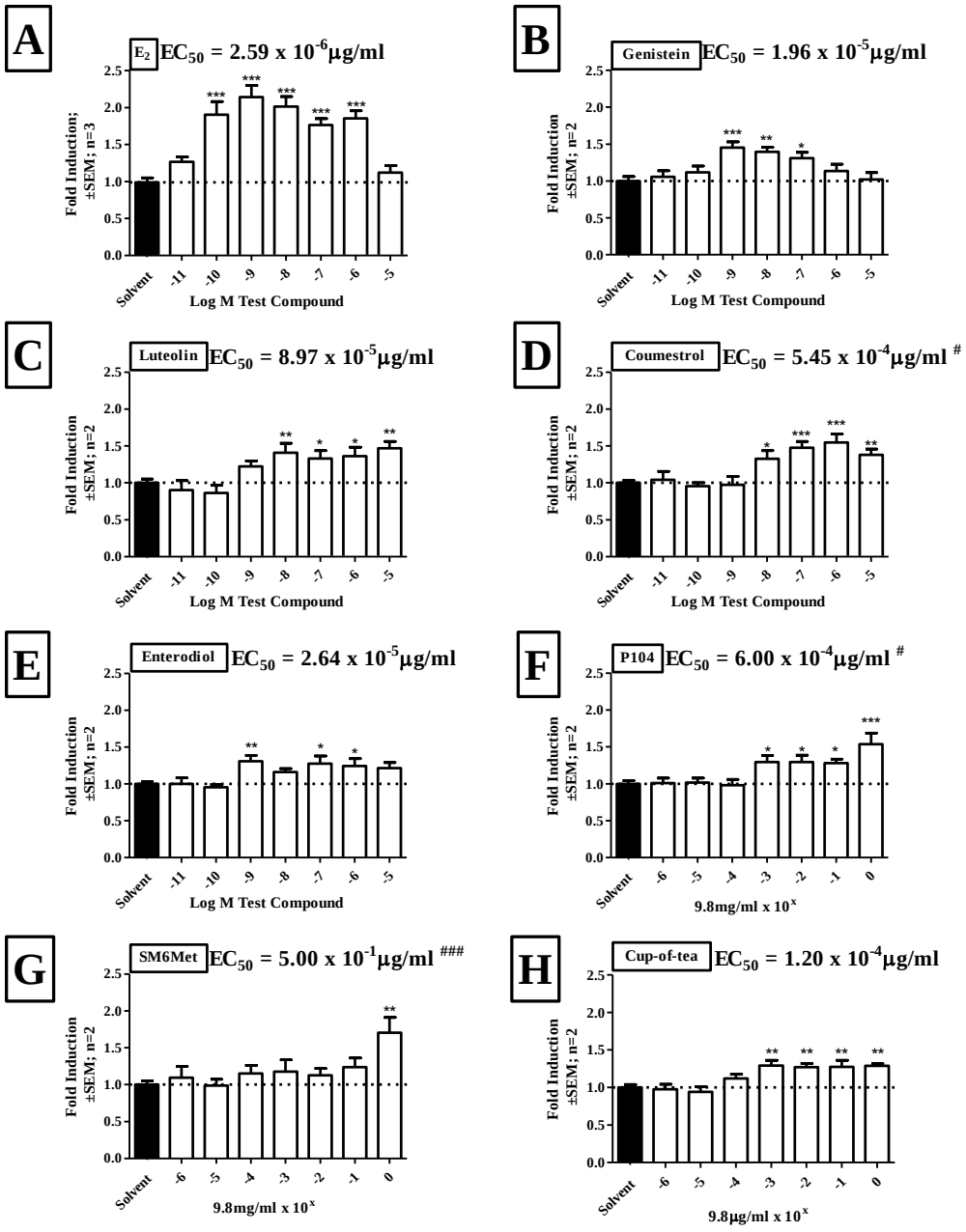


Figure 5. Evaluation of agonism of proliferation, a more complex endpoint encompassing both transactivation and transrepression in MCF-7BUS cells expressing both ERα and ERβ. MCF-7 BUS cells were treated with increasing concentrations of (A) E_2, (B-E) polyphenols, and (F-H) *Cyclopia* extracts for 48 hours. After treatment the amount of living cells was determined using a MTT assay. Statistical analysis was done using One-way ANOVA with Dunnett's post-test comparing all columns to the solvent control (*, P<0.05; **, P<0.01; ***, P<0.001) or to E_2 for EC_{50} values (#, P<0.05; ##, P<0.01; ###, P<0.001). The dotted line through the bars represents the values for solvent control. Mean ± SEM is of two independent experiments done in six replicates, except (A) where mean ±SEM is of three independent experiments done in six replicates.

The potencies, depicted by EC_{50} values on graphs (Figs. 5A-H), of the polyphenols, as well as of the *Cyclopia* extracts, were lower than that of E_2 with coumestrol, P104, and SM6Met significantly

lower and may be listed in order of decreasing potency as follow: E_2 > genistein > enterodiol > luteolin > cup-of-tea > P104 > coumestrol >> SM6Met.

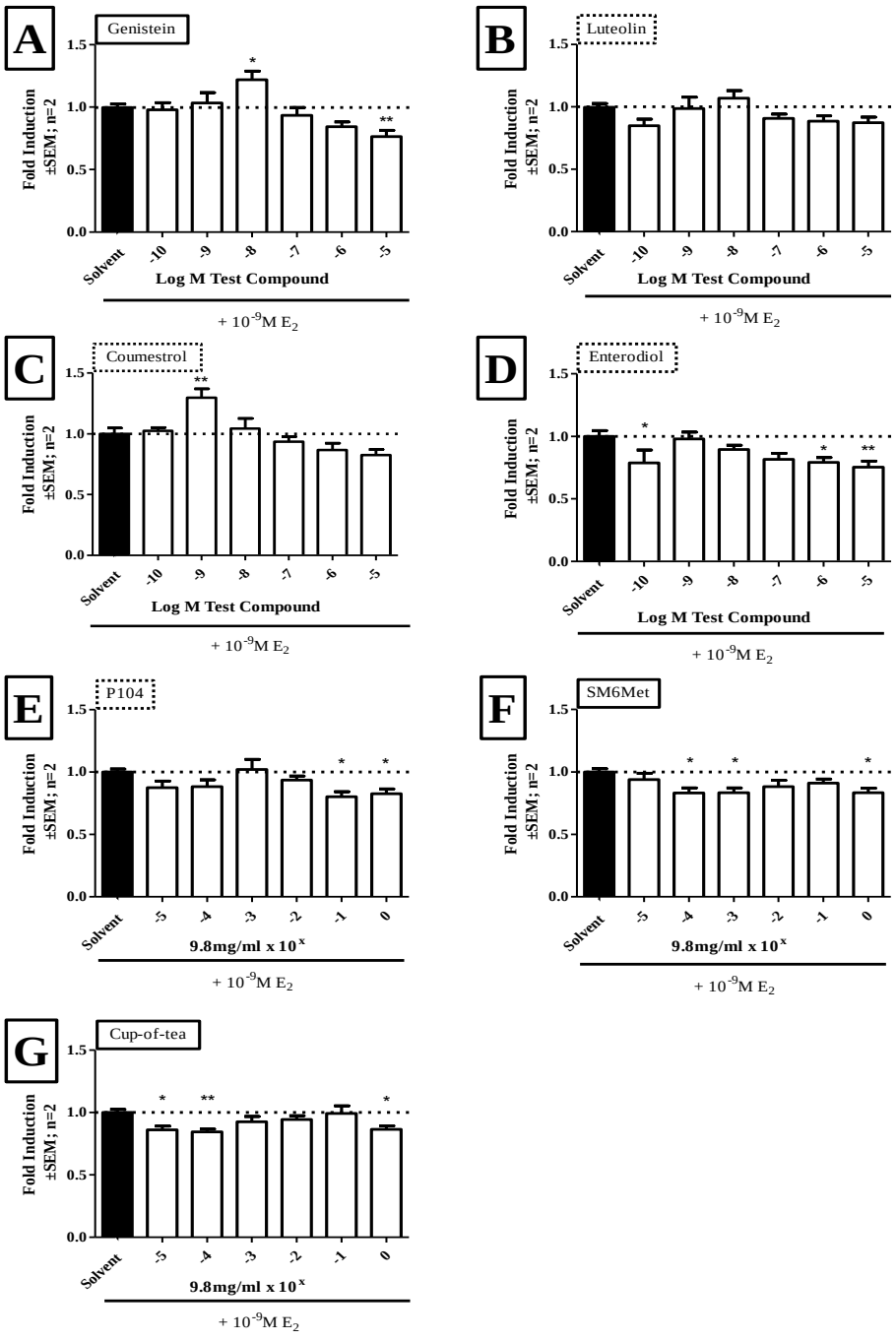


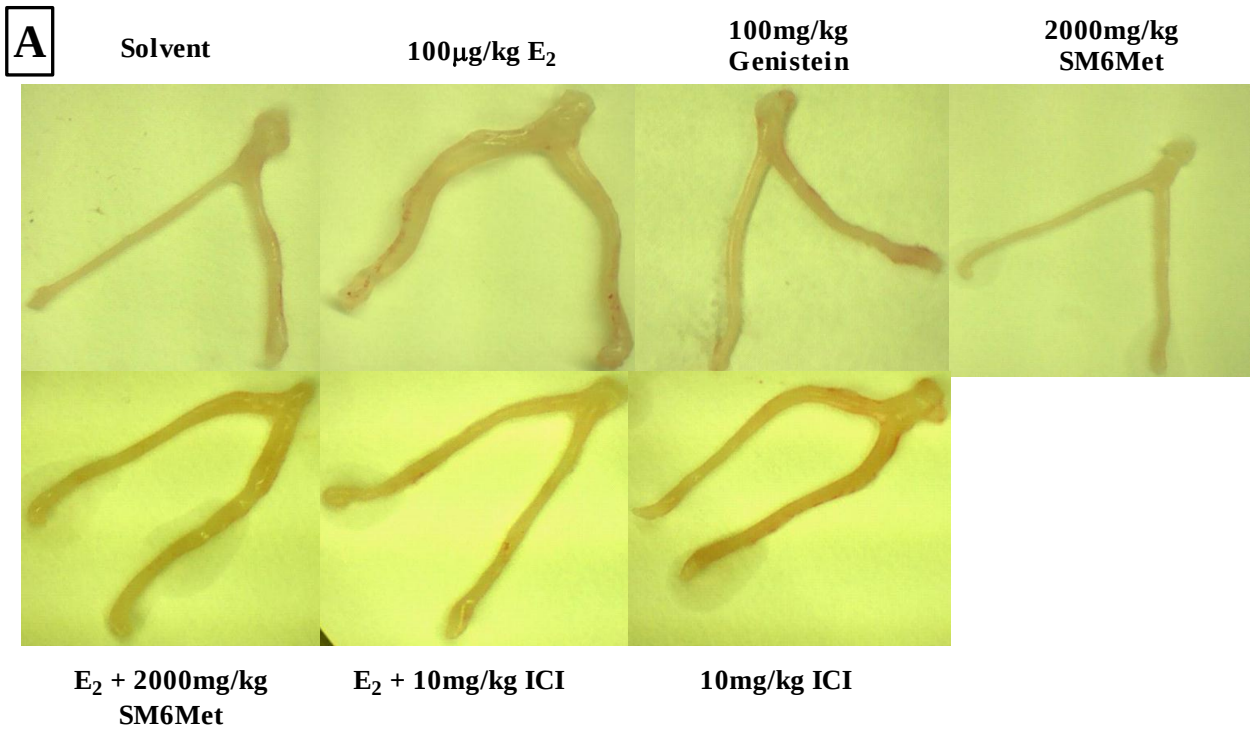
Figure 6. Evaluation of antagonism of proliferation, a more complex endpoint encompassing both transactivation and transrepression in MCF-7BUS cells expressing both ERα and ERβ. MCF-7 BUS cells were treated with increasing concentrations of (A-D) polyphenols and (E-G) *Cyclopia* extracts for 48 hours in the presence of 10^{-9}M E_2. After treatment the amount of living cells was determined using a MTT assay. Statistical analysis was done using One-way ANOVA with Dunnett's post-test comparing all columns to the solvent control (*, P<0.05; **, P<0.01; ***, P<0.001). The dotted line through the bars represents the values for solvent control. Mean ± SEM is of two independent experiments done in six replicates.

To address antagonism (Fig. 6A-G), increasing concentrations of the polyphenols and *Cyclopia* extracts were tested in the presence of 10^{-9} M E_2 (highest efficacy, Fig. 5A). Genistein (Fig. 6A) and enterodiol (Fig. 6D), significantly repressed E_2-induced cell proliferation (23.3% at 10^{-5} M (2.70 μg/ml) and 24.5% at 10^{-5} M (3.02 μg/ml), respectively). Although, luteolin (Fig. 6B) and coumestrol (Fig. 6C) displayed no significant antagonism, coumestrol did have a significant additive effect (1.3 ± 0.1 fold) at 10^{-9} M (2.96 x 10^{-4} μg/ml), suggesting agonism. Similarly, genistein, an antagonist at high concentrations, also had a significant additive effect (1.2 ± 0.1 fold) at the lower concentration of 10^{-8} M (2.70 x 10^{-3} μg/ml) (Fig 6A). All extracts of *Cyclopia* were able to antagonize E_2-induced cell proliferation, with P104 repressing 19.8% at 9.8 x 10^{-1} μg/ml, SM6Met 16.8% 9.8 x 10^{-4} μg/ml, and cup-of-tea 15.6% repression at 9.8 x 10^{-4} μg/ml (Figs. 6E, F, G). Taken together, these results show that although all extracts of *Cyclopia* induced cell proliferation, the P104 and cup-of-tea extracts did so at a significantly lower efficacy and the methanol extracts at a significantly lower potency than E_2, and that all extracts could antagonize E_2-induced cell proliferation.

3.3.7. SM6Met does not stimulate the growth of rat uteri, antagonizes E_2-induced uterine proliferation, and delays vaginal opening.

For the *in vivo* studies only extracts from *C. subternata* was used as P104 plant material was not available in bulk. The immature rat uterotrophic assay is used to determine the ability of test compounds to stimulate ERα induced uterine growth as ERβ is not highly expressed in the uterus [56,101] and also allows for the detection of antiestrogens [102]. Rats were administered E_2, genistein, and the two *C. subternata* extracts, SM6Met and cup-of-tea, via oral gavage and the effects on uterine growth were evaluated (Fig. 7A, B, and Fig. S3). Estrogen, as well as genistein, induced uterine growth (2.5 ± 0.2 and 2.0 ± 0.2 fold, respectively) (Fig. 7). In contrast, the extracts significantly reduced uterine weight relative to solvent (Fig. 7 and Fig. S3). SM6Met also significantly repressed E_2-induced uterine growth by 33.0%, a result that is similar, but less

pronounced, than that seen with ICI 182,780 (59.7% repression) (Fig. 7) suggesting that the extracts behave as antiestrogens in the uterus.



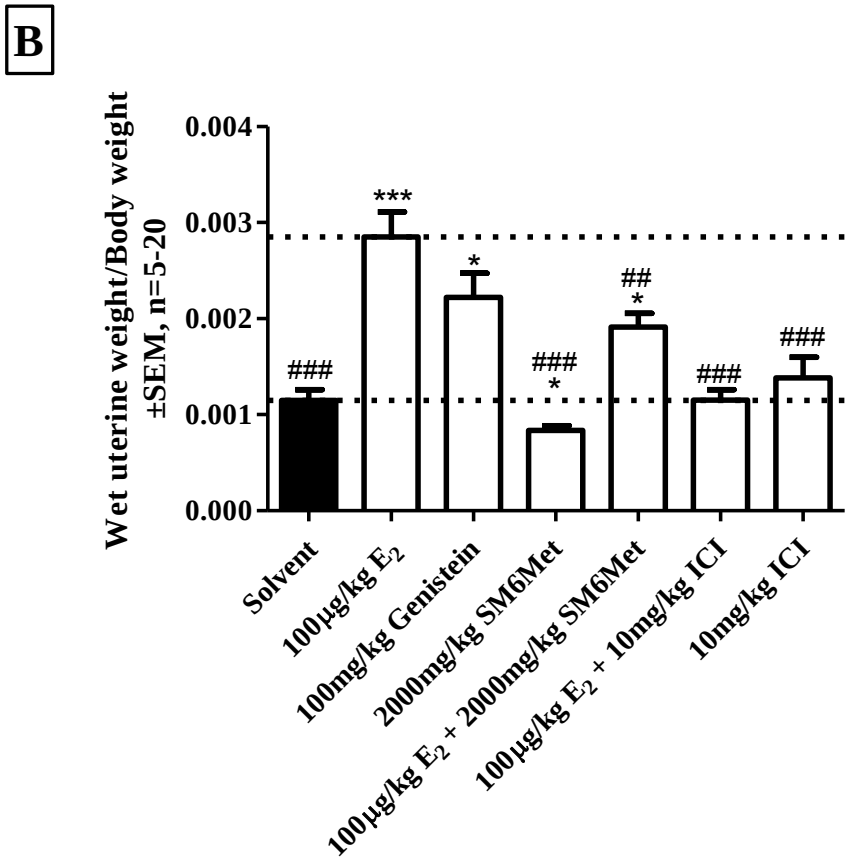


Figure 7. Evaluation of the *in vivo* effect of E_2, genistein and SM6Met on immature rat uterine growth. Immature female wistar rats were treated with 100µg/kg body weight E_2, in the presence and absence of 2000mg/kg body weight SM6Met or 10mg/kg body weight ICI 182,780, 100mg/kg body weight genistein, 2000mg/kg body weight SM6Met, and 10mg/kg body weight ICI 182,780 for three consecutive days. Animals were sacrificed on day four, (A) uteri were photographed, and (B) wet uterine/final body weight was determined. One-way ANOVA with Dunnett's post-test comparing all columns to either solvent control (*, P<0.05; **, P<0.01; ***, P<0.001) or E_2 (#, P<0.05; ##, P<0.01; ###, P<0.001). The dotted lines through the bars represent the values for solvent control or E_2. Mean ± SEM is of at least five animals/group.

We also addressed body weight changes and toxicity (Fig. S4) and found that E_2 significantly increased body weight, whereas genistein significantly decreased body weight. The extracts of *Cyclopia* and ICI 182,780, however, did not lead to significant weight gain or loss as compared to solvent, except for the animals treated with the highest concentrations (2000mg/kg BW) of SM6Met and cup-of-tea extracts which gained significantly less weight than the solvent treated animals. With regards to toxicity, none of the treated animals showed any significant changes in liver weight, except for a decrease in liver weight in animals treated with 200mg/kg BW SM6Met.

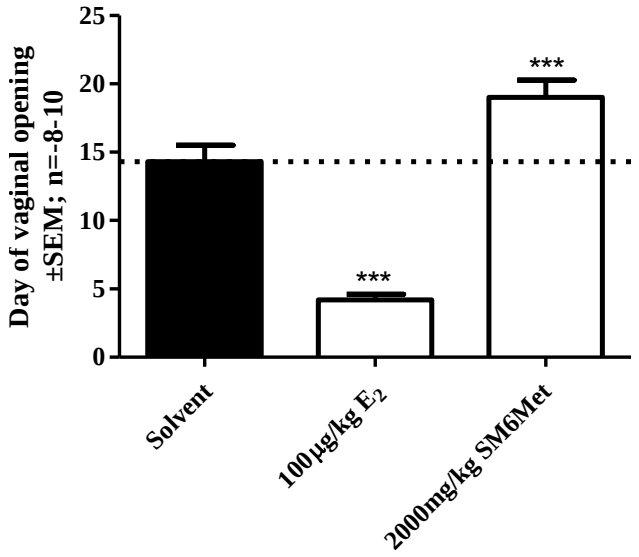


Figure 8. Evaluation of the effect of E_2 and SM6Met on the timing of vaginal opening. Immature female wistar rats were treated for 30 consecutive days with 100µg/kg body weight E_2 and 2000mg/kg body weight SM6Met and the day of vaginal opening was determined. One-way ANOVA with Dunnett's post-test comparing all columns to solvent control (*, P<0.05; **, P<0.01; ***, P<0.001). The dotted line through the bars represents the values for solvent control. Mean ± SEM is of at least eight animals/group..

Furthermore, as another marker of estrogenic activity, albeit a less sensitive marker [102], we also evaluated time of vaginal opening over an extended period of daily treatments (Fig. 8). Estrogen led to premature vaginal opening when compared to solvent (4.2 ± 0.4 vs. 14.3 ± 1.2 days). This

correlates with the observed increase in uterine weight in Figure 7. The significantly delayed vaginal opening of SM6Met treated animals (19.0 ± 1.3 days) also correlates with uterine weight results in displaying antiestrogenic behavior. The significant delay in vaginal opening was observed for all three of the concentrations of SM6Met, however, although the cup-of-tea extract showed a similar trend, it was not significant (Fig. S5).

To summarize, for the first time we show that the *C. subternata* extracts are absorbed when administered orally and elicit a biological effect *in vivo*. Specifically, *Cyclopia* extracts, in contrast to E_2 and genistein, did not induce uterine growth and SM6Met antagonized E_2-induced uterine growth. Furthermore, the extracts also delayed vaginal opening in contrast to E_2. These results suggest that the *Cyclopia* extracts display ERα antagonism *in vivo* by retarding uterine growth [56,101].

3.4. Discussion

HRT in the form of estrogens provides relief from the plethora of menopause associated symptoms [1]. Although these estrogens provide relief from menopausal symptoms, they introduced new HRT associated risks, including an increased occurrence of breast cancer, heart disease, strokes, and endometrial cancer [1,5,6,8]. These risks, and the associated reluctance of usage, instigated the search for a new generation of estrogen analogues that would provide the benefits of estrogens without the associated risks. In addition, it would be of great value if these new analogues display chemo-preventative properties in breast and endometrial tissues [9,10,29].

The search for new estrogen analogues heralded the era of the SERMs. These SERMs would selectively modulate estrogen receptors in different tissues, acting as antagonists in the breast and uterus (chemo-preventative) and as agonists in the bone (osteoporosis prevention). Tamoxifen, a first generation SERM, provided the desired protective effect in the breast [31,32] and raloxifene, a second generation SERM, had protective properties in breast and bone tissues [26,27,103]. However, as these SERMS have been linked to the increased occurrence of hot flashes and stimulated endometrial growth (tamoxifen), the search continues [28,34,35]. Third generation

SERMs, such as lasoxifene and bazedoxifene, are currently in development, but the focus has shifted to osteoporosis treatment with protection against breast cancer as a beneficial side effect [104-106].

Although SERM development continues there is increased interest in SERSMs, analogues that can differentially modulate specific ER subtypes. This was brought on by studies that have shown that ERβ inhibits ERα dependent cell proliferation and could prevent cancer development [15,22,37,40-43]. Phytoestrogens have been shown to be both estrogenic as well as antiestrogenic and while they can bind to both ER subtypes, they generally have a higher affinity for ERβ as well as a higher transcriptional potency and efficacy via ERβ [61-63]. Thus, phytoestrogen rich food sources have become important potential resources of SERSMS.

The current study evaluated previously described extracts of *Cyclopia*, a source of phytoestrogens, for ER agonism and/or antagonism (summarized in Table S1). Specifically, we evaluated the effect of *Cyclopia* extracts on transactivation and transrepression in a model where ERα and ERβ were expressed separately. This allows for the evaluation of the modulation of ER subtype specific activity in two transcriptional models: a classical ERE transactivation model and an NFκB transrepression model. In the transactivation model the methanol extracts, P104 and SM6Met were ERβ agonists, while all extracts antagonized ERα. In the transrepression model, however, the behavior of the *Cyclopia* extracts became more complex. P104, which displayed opposite effects via the subtypes in the transactivation model, acted as an agonist for both subtypes in the transrepression model. The extracts of *C. subternata*, however, did not elicit such uniform effects in the transrepression model. SM6Met, a methanol extract, acting as an ERα antagonist and ERβ agonist regarding transactivation, displayed antagonism towards ERα, in the absence of E_2, and towards ERβ, in the presence of E_2. Similar antagonism towards ERα in the absence of E_2 has also been seen for the plant extract MF101 regarding IL6 mRNA expression [24]. The water extract, cup-of-tea, also changed its behavior, acting as an ERβ antagonist for transrepression as opposed to an ERα antagonist for transactivation. These behavioral changes were not exclusive to the *Cyclopia*

extracts as the polyphenols also displayed these characteristics. Luteolin, for example, displayed ER agonism through both subtypes in the transactivation model but was an ERα agonist and an ERβ antagonist in the transrepression model. The occurrence of mixed agonism and antagonism towards ER subtypes has also been observed for the xenoestrogen, Bisphenol A (BPA) [107].

As the current experiments were performed in the same cell line we have to look towards differences between the mechanisms of transactivation and transrepression for clarification of these results. Classically, transactivation is a product of ER dimer binding directly to the DNA sequence, however, tethering to DNA bound transcription factors (TFs) in the promoter region of affected genes has also been described [108-111]. Binding of the ER to DNA, whether it is direct or indirect, initiates the recruitment of co-activators, which then modulates transcription [112]. Regarding transrepression, specifically the repression of NFκB driven genes, various mechanisms of ER mediated transrepression have been described [109]. The ER can bind to NFκB and thereby prevent DNA binding of the transcription factor [113,114], ligand bound ER present at promoter regions can recruit co-repressors [115,116], ligand bound ERα and activated NFκB can compete for co-activator recruitment [117,118], or ERα, through a non-genomic pathway, inhibits translocation of activated NFκB to the nucleus [119]. We can use this knowledge of the mechanism of action and combine it with what we know about SERMs and ER subtypes specific ligands to postulate a mechanism of action of *Cyclopia* agonism and antagonism. For the SERMs, three mechanisms of antagonism have been proposed [18]. SERMs can bind to the ER with a higher affinity than E_2 and block the binding of E_2, they can block the binding of co-activators, or SERMs can induce the recruitment of co-repressors. [18,120,121]. Not much is known regarding the mechanism of SERM agonism [18], although it has been suggested that they can block the binding of co-repressors [121]. In addition, MF101 and liquiritigenin, both ERβ selective agonists, although being able to bind to ERα, cannot recruit co-activators to ERα, and MF101 cannot promote the interaction of ERα with regulatory elements [15,24]. Furthermore, it has been suggested that SERMs may activate cell surface signaling pathways that results in ligand-independent activation of ERs [29,122,123].

Therefore, with regards to transactivation, we may postulate that the extracts of *Cyclopia* cannot transactivate via ERα as they are unable to recruit the necessary co-activators, while for ERβ, P104 and SM6Met are able to do so. It is also possible that the extracts of *Cyclopia* cannot induce ERα interaction with regulatory elements. The observed ERα antagonism of E_2-induced transactivation may be due to the extracts binding to ERα and either inhibiting E_2 binding, inhibiting the recruitment of co-activators or stimulating the recruitment of co-repressors. In our transrepression model P104 behaves like E_2 and could be exerting its function by any of the NFκB repression models discussed earlier. However, SM6Met displays ERα antagonism in the absence of E_2 and this antagonism is lost in the presence of E_2. Therefore, it is possible that SM6Met is unable to recruit co-repressors in the absence of E_2 and is unable to inhibit the E_2-induced recruitment of co-repressors. Furthermore, antagonism of ERβ in the transrepression model by SM6Met and cup-of-tea may be due to the recruitment of co-activators to ERβ.

Next we evaluated agonism and antagonism of *Cyclopia* extracts in a more complex environment where the ER subtypes are co-expressed. We used the MCF-7BUS cells, a breast carcinoma cell line, not only because it co-expresses the subtypes (Fig. 2A), but also to evaluate the activity of the extracts in breast tissue cells. With regards to transactivation, all extracts of *Cyclopia* were agonists and are likely exerting this agonism through ERβ as they were ERβ agonists and ERα antagonists in COS-1 cells. Also, previously we discussed the possibility that the extracts may be unable to recruit co-activators to ERα or induce ERα-regulatory element interactions, which supports the idea that the *Cyclopia* extracts are mediating their transactivative effects in MCF-7BUS cells via ERβ. Interestingly, the polyphenols, genistein and luteolin, having displayed ER agonism in COS-1 cells, in an environment where both ER subtypes are present displayed only weak agonism, which may be attributed to the fact that lower concentrations were used in MCF-7BUS cells. However, when both subtypes are present these polyphenols display antagonism, which was not apparent when the subtypes were expressed separately. When both ER subtypes are expressed in the transrepression model, all the polyphenols as well as the *Cyclopia* extracts acted as agonists, while the water extract

of *C. subternata* also displayed ER antagonism. The ER agonism of P104 in the transrepression model is thus not a cell type selective effect as it is seen in both the COS-1 (kidney) and MCF-7BUS (breast) cells. The ER antagonism of cup-of-tea in MCF-7BUS cells is likely mediated via ERβ as ERβ antagonism was observed in COS-1 cells transfected with ERβ, but not in cells transfected with ERα. However, the SM6Met extract, which displayed antagonism for ERα and ERβ in COS-1 cells, changes its behavior in the MCF-7BUS cells and acts as an ER agonist in the transrepression model. Furthermore, a similar switch in behavior is observed with the polyphenols as the subtype specific antagonism is abrogated in the presence of both ER subtypes. These observed behavioral changes of the *Cyclopia* extracts as well as the polyphenols in different tissues have also been observed for the SERM, tamoxifen [18]. Ball *et al.* [18] found that tamoxifen differentially regulated ER regulated genes in different cell lines and ascribed this phenomenon to the presence, or lack of, co-regulators in different tissues. Therefore, the differential effect of *Cyclopia* extracts as well as the polyphenols in cells from different tissues might be due to changes in the co-regulator environment.

As MCF-7BUS cells express both ER subtypes, we also have to consider the possibility of ERα/β heterodimer formation and the biological relevance thereof as opposed to homodimer formation in COS-1 cells expressing the ER subtypes in isolation. Using two phytoestrogens that are ERα/β heterodimer selective, cosmosiin and angolensin, it was shown that heterodimer formation, in the presence of these ligands, leads to higher activation of an ERE-promoter reporter construct than homodimers and furthermore that heterodimer formation has a growth inhibitory effect in breast and prostate epithelial cells [124]. Previous studies by Powell *et al.* [46] showed that the ERβ selective agonist, liquiritigenin, which can bind to both ER subtypes, induces an ERα conformation that prefers heterodimerization with ERβ, as opposed to forming ERα homodimers. Therefore, we cannot exclude heterodimer formation as an explanation for the strong agonist effect of the *Cyclopia* extracts in the transactivation model in MCF-7BUS cells.

Having evaluated the agonist and antagonist activity of *Cyclopia* extracts in a system where the ER subtypes were expressed separately and together, in a transactivation and a transrepression model, we increased the level of complexity by evaluating the effect of the extracts on MCF-7BUS cell proliferation, a system where the final cell phenotype is a product of not only the two ER subtypes but also of an integrated transactivation and transrepression system [39,98-100]. Although the *Cyclopia* extracts, like E_2, induced cell proliferation it was with either a significantly lower potency (P104 and SM6Met) or lower efficacy (P104 and cup-of-tea) than E_2. Furthermore, in the presence of E_2, all of the *Cyclopia* extracts displayed antagonistic properties. Similarly, the polyphenols also induced cell proliferation with either lower efficacies or potencies than E_2 and some (genistein and enterodiol) also displayed antagonism. Previously, the agonist activity seen in the transactivation model in MCF-7BUS cells was ascribed to ERβ activation and this is probably translating into weak induction of MCF-7BUS cell proliferation. Furthermore, liquiritigenin, an ERβ selective agonist, although not able to induce significant MCF-7 cell growth in a mouse xenograft model [19,24], was able to induce proliferation of the ERα and ERβ positive [125] osteoblast-like murine MC3T3-E1 cells [126]. The antagonism of E_2-induced cell proliferation by extracts of *Cyclopia* could be attributed to ERα antagonism (observed in the transactivation model in COS-1 cells), ER mediated repression of proliferation inducing genes (ER transrepression observed in MCF-7BUS transrepression model), ERβ-mediated transcription (observed in the transactivation model in COS-1 cells) of anti-proliferative and anti-apoptotic genes [39,127], or they might favor the formation of ERα/β heterodimers, which has been suggested to have growth inhibitory effects in breast epithelial cells [124].

Furthermore, we also evaluated the estrogenic and antiestrogenic properties of the *Cyclopia* extracts in an *in vivo* model, an immature rat uterotrophic assay. For the first time we show *in vivo* biological activity of the phytoestrogenic extracts of *Cyclopia*. SM6Met and cup-of-tea, unlike E_2 and genistein, did not increase uterine weight and SM6Met, like the ER antagonist ICI 182,780, antagonized E_2-induced uterine growth. The ERα subtype is the major subtype expressed in the

uterus with very low levels of ERβ expressed [56,101]. Powell *et al.* [46] show that although ERβ homodimers and ERα/ERβ heterodimers are favored, genistein is capable of inducing ERα homodimers and activating ERα-induced transcription. Therefore, we can assume that the increase in uterine growth induced by genistein in the uterotrophic assay is a product of increased ERα homodimerization and hence, increased ERα mediated transcription. The ERβ selective agonists, liquiritigenin and ERB-041, in contrast, do not induce uterine growth [19,128]. Thus, the findings regarding ERβ selective agonists combined with the low levels of ERβ in the uterus excludes ERβ as the subtype eliciting the effect of *Cyclopia* extracts in the uterus. It is thus likely that the effect of *Cyclopia* extracts is due to ERα antagonism, as seen in the transactivation model in COS-1 cells, or that upon binding to the ER, the *Cyclopia* extracts induce a change in conformation that inhibits co-activator recruitment or activates co-repressor recruitment. The inability of the *Cyclopia* extracts to induce uterine growth, in contrast to MCF-7BUS cell proliferation, might also be attributed to either the differences in the concentration of co-regulators or the differences in co-regulator recruitment in the breast and uterus [129,130].

Having established ER agonist and/or antagonist activity of *Cyclopia* extracts, we look towards HPLC data, from the current and previous studies, to identify the polyphenol(s) responsible for the observed effects. The xanthones, mangiferin and isomangiferin, were identified in all *Cyclopia* extracts, but as mangiferin has no estrogenic potential, while isomangiferin has not previously been tested for estrogenicity [71], it is unlikely that the observed ER agonist/antagonist effects of *Cyclopia* can be ascribed to these polyphenols. However, mangiferin has been shown to inhibit the proliferation of breast cancer cells via ER independent mechanisms [131] and therefore, as mangiferin is present in all extracts at relatively high amounts it cannot be excluded as the polyphenol antagonizing E_2-induced MCF-7BUS cell proliferation. Of the remaining polyphenols identified in the extracts the only aglycone present is the flavone, luteolin. *In vitro*, luteolin binds to both of the ER subtypes, is an ERα and ERβ agonist, induces MCF-7BUS cell proliferation, and antagonizes E_2-induced MCF-7BUS cell proliferation [62,63,71,132-134]. Therefore, with regards

to the *Cyclopia* extracts, the ERβ agonism observed in the transactivation model, the induction of MCF-7BUS cell proliferation, and the antagonism of E_2-induced cell proliferation may be ascribed to the presence luteolin in the extracts, however, the observed ERα antagonism in the transactivation model cannot. Although luteolin is present in all extracts, the concentration is low. However, the 7-*O*-rutinoside of luteolin, scolymoside, is present in substantial amounts in all of the *C. subternata* extracts (presence was not evaluated in P104). This rutinoside of luteolin has not previously been tested for estrogenicity [71], however, as glycosides may be hydrolyzed by intestinal β-glucosidases [135,136], the bioavailability of the aglycone, luteolin, and hence phytoestogenicity of the extracts may increase upon hydrolysis of scolymoside. Furthermore, luteolin has been shown to have anti-tumor characteristics and can sensitize breast cancer cells to anti-tumor drugs such as tamoxifen [137] and therefore, the presence of luteolin, as well as scolymoside, in *Cyclopia* extracts can be seen as positive regarding chemoprevention as well as breast cancer treatment. Generally, the glycosides of polyphenols either display reduced estrogenic activity compared to the aglycones or have not been evaluated for estrogenicity [71]. Thus, if the hydrolysis of glycosides present in the *Cyclopia* extracts is considered, it allows us to evaluate the phytoestrogenicity of the aglycones alongside their glycosides: apigenin (aglycone of vicenin-2), eriodictyol (eriocitrin), hesperitin (hesperidin), phloretin (phloretin-3,5-di-*C*-glucoside), hydroxyphloretin (3-hydroxyphloretin-3',5'-di-*C*-hexoside), and iriflophenone (iriflophenone-2-*C*-β-glucoside and iriflophenone-di-*O*,*C*-hexoside). However, as β-glucosidases are produced by intestinal flora [138,139], consideration of glycoside metabolism will not help to identify the polyphenols responsible for *in vitro* results but may only be relevant for interpretation of *in vivo* results. For example, as luteolin and apigenin have been shown to significantly increase uterine weight, either in the presence or absence of estrogens [140,141], the effect elicited by *Cyclopia* extracts *in vivo* cannot be ascribed to luteolin, scolymoside, or vicenin-2. The effect of the other identified polyphenols has not been evaluated *in vivo* and therefore we cannot definitively attribute the *in vivo* effect of the *Cyclopia* extracts to any of these polyphenols. Of the glycosides, ericotrin

and hesperidin have been tested for phytoestrogenicity *in vitro* [71]. However, hesperidin does not bind to the ER [62] or activate an ERE-containing promoter reporter construct [133]. Eriocitrin, however, has been shown to bind to only ERβ [62], but no work has been done to elucidate the estrogenic effect elicited by this polyphenol. The dihydrochalcone, 3-hydroxyphloretin-3',5'-di-*C*-hexoside, has previously been identified in *Cyclopia* [75]. 3-Hydroxyphloretin-3',5'-di-*C*-hexoside has not been tested for estrogenicity, but aspalathin, a monoglucoside, has been shown to inhibit the proliferation of liver cells [142], however, due to the presence of unique drug metabolizing enzymes in the liver, the possibility of aspalathin metabolites eliciting this effect cannot be excluded nor can the results be extrapolated to breast cancer cells. The phytoestrogenicity of the remaining glycosides and aglycones, as well as protocatechuic acid, has not been tested [71]. In summary, none of the compounds identified in the *Cyclop*ia extracts can account for the observed ERα antagonism, some (luteolin and eriocitrin) may explain the observed ERβ agonism and others (mangiferin and aspalathin) should not be excluded as possible effectors of ER-independent effects on proliferation. Therefore, thus far, we cannot with certainty ascribe the effects observed with *Cyclopia* extracts in this study to any of the individual constituents of our extracts. Although, further research regarding the polyphenol content, bioavailability, and estrogenic activity of our extracts is required to identify the compound causing the observed effects, we cannot exclude the possibility that a mixture of polyphenols is required to elicit the effects observed with *Cyclopia* extracts.

Physiologically, our results may be assessed both in terms of treatment of menopausal symptoms (hot flashes, osteoporosis, and increased inflammation [2-4,52,90-92]) and prevention of estrogen replacement associated side effects (breast cancer and uterine proliferation [5,6,52]). With regards to menopausal symptoms, the ERβ agonist MF101 [24], has been shown in clinical trials to reduce hot flashes and thus, the ERβ agonism of the *Cyclopia* extracts may be considered as a positive attribute. Furthermore, with regards to the postmenopausal surge in inflammatory disorders the fact that the *Cyclopia* extracts displayed agonism in the transrepression model in MCF-7BUS cells may

also be considered as a positive attribute for the treatment of postmenopausal inflammatory disorders. With respect to the known roles of ER subtypes in breast cancer [15,22,37-43], the fact that extracts of *Cyclopia* antagonize ERα, while being ERβ agonists, may be beneficial. In addition, the extracts were able to antagonize the proliferation of breast cancer cells in the presence of E_2 at lower concentrations than that required for breast cancer cell proliferation. Furthermore, not only do the *Cyclopia* extracts show potential as protectors against breast cancer development and inflammatory disorders, they also do this without promoting uterine growth, a negative SERM associated side effect [35,143].

Although *Cyclopia* extracts show potential to be developed as SERSMs, further work, which is ongoing, is needed to clarify their mechanism of action. This includes, but is not limited to, directly comparing the *Cyclopia* extracts with the known SERMs tamoxifen and raloxifene, investigating the effect of *Cyclopia* extracts on ER subtype levels, ER homo- or heterodimerization, induction or inhibition of co-regulator recruitment, and the modulation of cancer development and progression in a rat breast cancer model. In addition, further work is needed to identify the polyphenol(s) responsible for eliciting the observed effects and the possibility that distinct polyphenols present in *Cyclopia*, rather than an individual polyphenol, may be causing the observed ERα agonism and ERβ antagonism cannot be excluded.

3.5. Literature cited

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3.5. Supporting Information

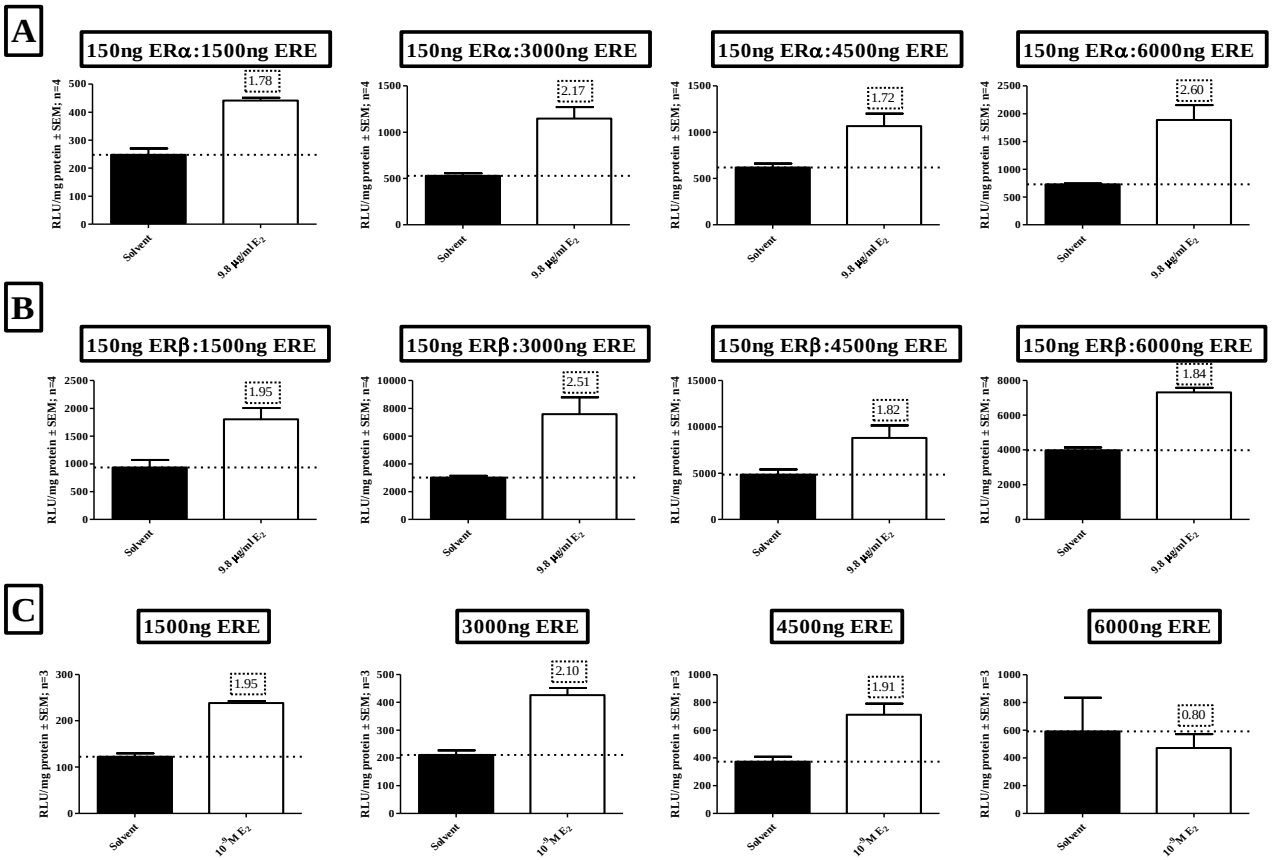

Figure S1. Determination of ERE-containing promoter reporter construct concentration. (A & B) COS-1 cells, transfected with equal amounts of (A) ERα and (B) ERβ, and (C) MCF-7BUS cells were transfected with increasing amounts of the ERE-containing promoter reporter construct (ERE.vit2.luc) and treated with either solvent or E_2 to determine at which concentration of the ERE-containing promoter reporter construct the highest induction of E_2 is observed. The dotted line through the bars represents the values for solvent control. Fold induction is indicated in boxes above the E_2 columns. Mean ± SEM is of one experiment done with three to four repeats.

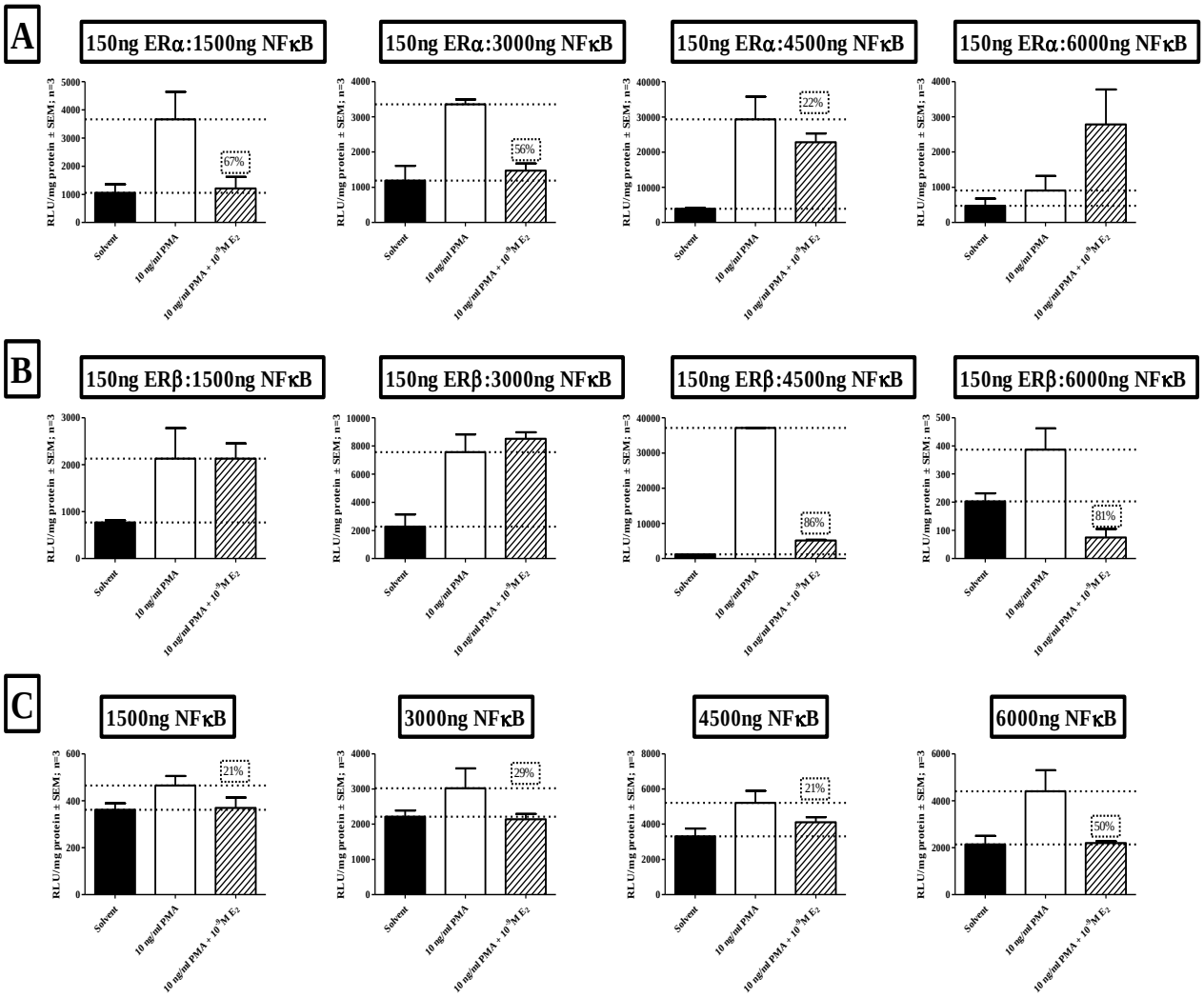

Figure S2. Determination of NFκB-containing promoter reporter construct concentration. (A & B) COS-1 cells, transfected with equal amounts of (A) ERα and (B) ERβ, and (C) MCF-7BUS cells were transfected with increasing amounts of the NFκB-containing promoter reporter construct (p(IL6kB)350hu.IL6Pluc+) and treated with either solvent, PMA or PMA + E_2 to determine at which concentration of the NFκB-containing promoter reporter construct the highest repression by E_2 of PMA induction is observed. The dotted lines through the bars represent the values for either solvent control or 10ng/ml PMA. Percentage repression, where applicable, is indicated in boxes above the PMA + E_2 columns. Mean ± SEM is of one experiment done with three repeats.

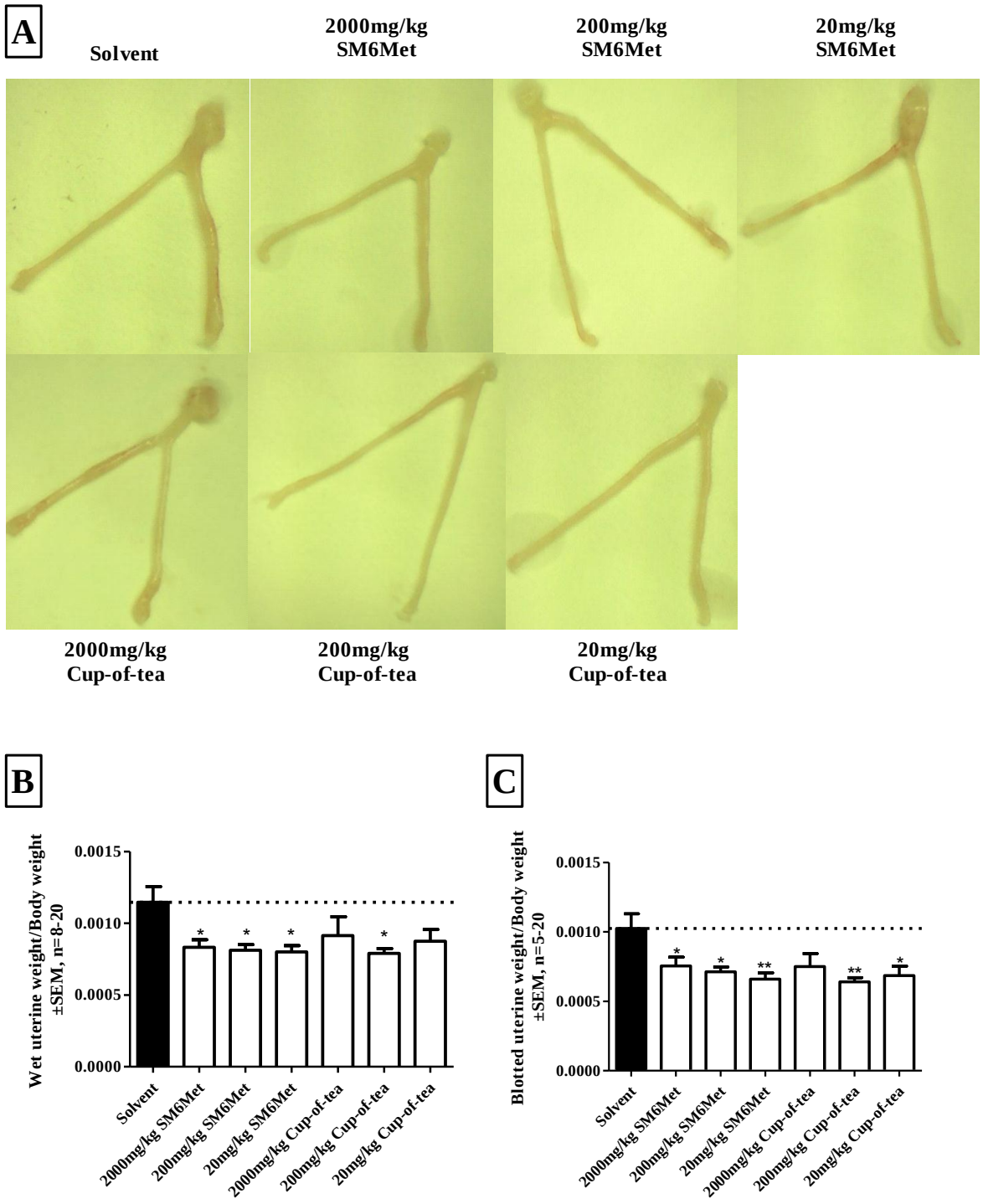

Figure S3. The effect of the SM6Met and cup-of-tea extracts on immature rat uterine growth. Immature female wistar rats were treated with 2000, 200, and 20mg/kg body weight SM6Met and cup-of-tea for three consecutive days. Animals were sacrificed on day four, (A) uteri were photographed and (B) wet and (C) blotted uterine/final body weight was determined. One-way ANOVA with Dunnett's post-test comparing all columns to solvent control (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$). The dotted line through the bars represents the values for solvent control. Mean ± SEM is of at least eight animals/group.

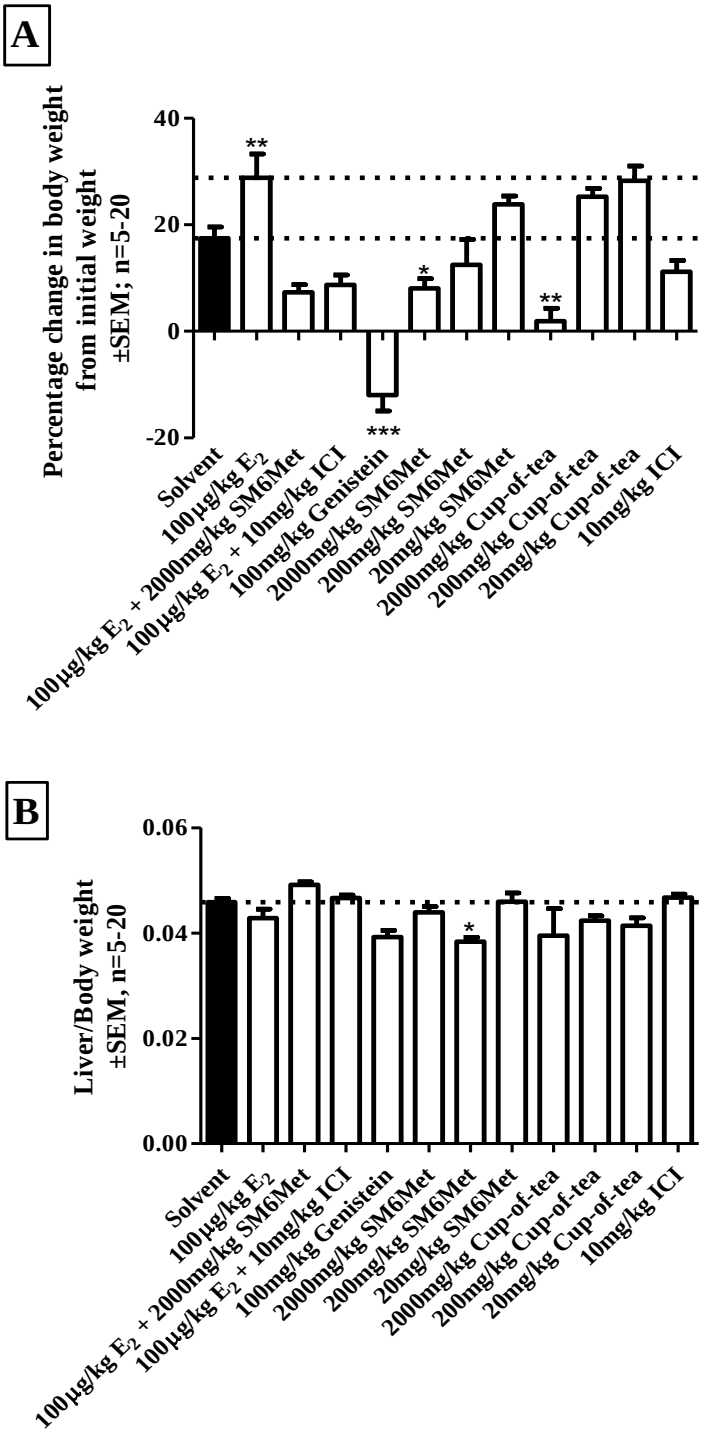

Figure S4. The effect of E_2, genistein, extracts of *Cyclopia*, and ICI on body and liver weight. Immature female wistar rats were treated for three consecutive days with 100µg/kg body weight (BW) E_2, in the presence and absence of 2000mg/kg BW SM6Met or 10mg/kg BW ICI 182,780, 100mg/kg BW genistein, 2000, 200, or 20mg/kg BW SM6Met, 2000, 200, or 20mg/kg BW cup-of-tea, and 10mg/kg BW ICI 182,780 for three consecutive days. Animal were sacrificed on day four and changes in (A) body and (B) liver weights were determined. One-way ANOVA with Dunnett's post-test comparing all columns to solvent control (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$). The dotted line through the bars represents the values for solvent control (A and B) and 100µg/kg BW E_2 (A). Mean ± SEM is of at least five animals/group

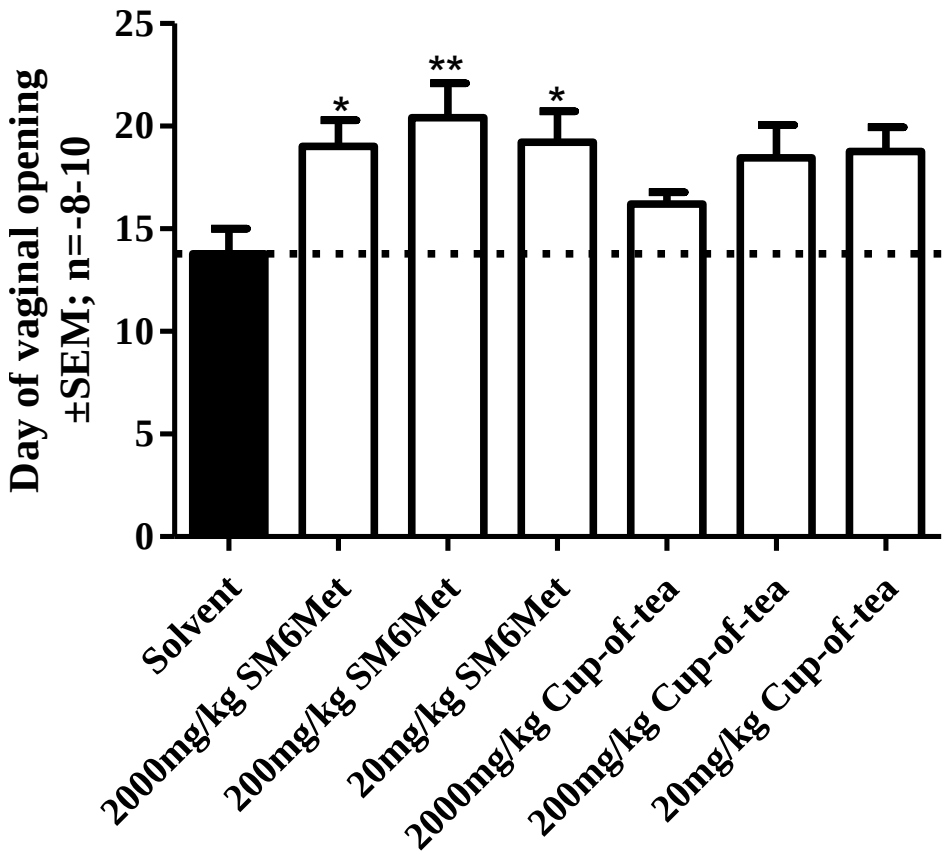

Figure S5. The effect of different concentration of the SM6Met and cup-of-tea extracts on the onset of vaginal opening. Immature female wistar rats were treated for 30 consecutive days with the SM6Met and cup-of-tea extracts and the day of vaginal opening was determined. One-way ANOVA with Dunnett's post-test comparing all columns to solvent control (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$). The dotted line through the bars represents the values for solvent control. Mean ± SEM is of at least eight animals/group

Table S1. Summary of ER agonism and antagonism of *Cyclopia* extracts.

Extract	Type of extract	Species	Test model	Agonist[a]	Antagonist[b]	Reference
P104	Methanol	*C. genistodes*	**Transactivation**: COS-1 cells + hERα/hERβ & ERE-promoter reporter construct.	ERβ	nt[c]	[1]
				ERβ	ERα	Fig. 1
			Transactivation: MCF-7BUS cells + ERE-promoter reporter construct.	ER	nt	Fig. 2
			Transrepression: COS-1 cells + hERα/hERβ & NFκB-promoter reporter construct.	ERα & ERβ	-[d]	Fig. 3
			Transrepression: MCF-7BUS cells + NFκB-promoter reporter construct.	ER	-	Fig. 4
			Cell proliferation: MCF-7BUS cells	ER	ER	[1]
				ER	ER	Figs. 5, 6
SM6Met	Methanol	*C. subternata*	**Transactivation**: ERα and ERβ expressing T47D-KBluc cells stably transfected with ERE-promoter reporter construct.	ER	nt	[2]
			Transactivation: COS-1 + hERα/hERβ & ERE-promoter reporter construct.	ERβ	ERα	Fig. 1
			Transactivation: MCF-7BUS + ERE-promoter reporter construct.	ER	-	Fig. 2
			Transrepression: COS-1 cells + hERα/hERβ & NFκB-promoter reporter construct.	-	ERα[e] & ERβ	Fig. 3
			Transrepression: MCF-7BUS cells + NFκB-promoter reporter construct.	ER	-	Fig. 4
			Cell proliferation: MCF-7BUS cells	ER	nt	[2]
				ER	ER	Figs. 5, 6
			Immature rat uterotrophic assay	-	ERα	Fig. 7
			Vaginal opening	-	ERα[f]	Fig. 8
Cup-of-tea	Water	*C. subternata*	**Transactivation**: COS-1 cells + hERα/hERβ & ERE-promoter reporter construct.	-	ERα	Fig. 1
			Transactivation: MCF-7BUS cells + ERE-promoter reporter construct.	ER	-	Fig. 2
			Transrepression: COS-1 cells + hERα/hERβ & NFκB-promoter reporter construct.	-	ERβ & weak ERα	Fig. 3

	Transrepression: MCF-7BUS + NFκB-promoter reporter construct.	ER	ER	Fig. 4
	Cell proliferation: MCF-7BUS cells	ER	nt	[2]
		Weak ER	ER	Figs. 5,6
	Immature rat uterotrophic assay	-	ERα	Sfig. 1
	Vaginal opening	-	Weak ERα	Sfig. 3

[a]Tested in the absence of E_2.
[b]Tested in the presence of 10^{-9} M E_2.
[c]nt = not tested
[d]- = no effect
[e]Tested in the absence of exogenous estrogens.
[f]Tested in the presence of endogenous estrogens.

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Chapter 4

Phytoestrogenic extracts of *Cyclopia* differentially targets ERα and ERβ protein levels and nuclear localization and distribution.

4.1. Introduction

Estrogens are sex hormones traditionally linked to the development of the female reproductive tract and secondary sex characteristics [1-3] and can affect cell viability, cell proliferation, and gene expression [4,5]. Estrogens exert their biological function by binding to the estrogen receptor (ER), a member of the nuclear receptor family of transcription factors, in the target organ/tissue [2,6,7]. The ER exists as two subtypes, namely ERα and ERβ [8].

Classically, upon estrogen binding, the ER undergoes a conformational change, dimerizes and binds to specific estrogen response elements (EREs), found in the promoter regions of estrogen responsive genes [2,4,5], however, tethering to DNA bound transcription factors (TFs) in the promoter region of affected genes has also been described [9-12]. The ER, even in the absence of ligand, is located primarily in the nucleus of the cell [13-16]. However, it has been shown that unliganded ERα shuttles from the nucleus to the cytoplasm [13]. Upon ligand activation both of the ER subtypes localize in the nucleus and form ordered clusters, which is indicative of areas of active transcription [16-18]. It has, however, been shown that different ER ligands can differentially affect both maximal nuclear localization and rate of nuclear localization [13,18]. Furthermore, different ER ligands have also been shown to result in dissimilar patterns of ER distribution within the nucleus, which correlates with different transcriptional outcomes [18,19].

The two ER subtypes, with opposing functions in breast cancer development and progression, adds to the complexity of ER signalling. Specifically, it has been shown that ERα is associated with cell proliferation and the occurrence of breast cancer, whereas several studies have shown that ERβ acts as an antagonist of ERα in breast cancer and could act as an inhibitor of breast cancer development [20-26]. Furthermore, although the two subtypes are co-expressed in approximately 60% of breast cancer tumours [27-29], higher ERα levels are associated with malignant tumours, while higher ERβ levels are

associated with benign tumours [25,30]. In addition, it has been suggested that tissue specific responses to ER agonists and antagonists may depend more on the relative levels of each subtype rather than absolute levels [31-33]. These findings suggest that evaluating the effects of treatment on the ratio of ERα:ERβ would be more useful than evaluating effects on the subtypes separately.

Although the ER subtypes complicate the understanding of ER signalling, it introduces an opportunity for novel drug development with the specific subtype protein levels as therapeutic targets. Advances towards the targeting of ER protein levels have been made. For example, the full ER antagonist, fulvestrant (ICI 182,780), a selective estrogen receptor degrader (SERD), promotes the degradation of ERα [34,35] while stabilizing ERβ protein levels [34]. Furthermore, fulvestrant inhibited the growth of breast tumour xenografts [36,37]. Unfortunately, during clinical trials, fulvestrant treatment caused undesirable side effects such as headaches, hot flushes, nausea, and disturbance of menses [38]. Although these are seen as minor side effects, the poor bioavailability, as well as length of time that it takes to achieve a useful therapeutic concentration in target tissues, eliminates fulvestrant in the search for the ideal breast cancer treatment [36,39]. GW5638/DPC974, an orally active non-steroidal tamoxifen derivative and SERD [36,39], also down-regulates ERα levels [39,40]. Although further development of GW5638/DPC974 was discontinued, afore mentioned findings merits further investigation regarding SERDs and the modulation of ER subtype specific regulation of physiological processes.

Extracts of *Cyclopia* (family: Fabaceae), an indigenous fynbos plant from the Western Cape province of South Africa [41,42], have previously been shown to bind to the ER and transactivate an ERE-containing promoter reporter construct [43-45]. Furthermore, in Chapter 3 we showed that these extracts elicit ER subtype specific responses by acting as ERα antagonists and ERβ agonists. Collectively these findings suggested that the *Cyclopia* extracts may be worthwhile candidates to

investigate for SERD activity, specifically, the ability of SERDs to down-regulate ERα [34,35,39,40] levels while stabilizing ERβ [35]. Therefore, in the MCF-7BUS cells, a human breast cancer cell line expressing both ERα and ERβ, we investigated the modulation by *Cyclopia* extracts of not only the ER subtypes levels individually, but also how these changes modified the ERα:ERβ ratio after treatment. As estrogen signalling is a product not only of estrogen binding to the ER, which is determined by the ER levels, but also ER nuclear localization [2,6,7,16,18], we in addition, using COS-1 cells transiently transfected with fluorescently tagged ER subtypes, investigated whether the *Cyclopia* extracts would alter the extent and rate of nuclear localization of the ER subtypes as well as nuclear distribution.

4.2. Material and methods

4.2.1. Test Compounds

17β-Estradiol (E_2), genistein, luteolin, enterodiol, and fulvestrant (ICI 182,780) were obtained from Sigma-Aldrich®, South Africa, and coumestrol was obtained from Fluka™ Analytical, Sigma-Aldrich®, South Africa. The *Cyclopia* extracts, P104 [44], SM6Met [45] and cup-of-tea [45], were previously prepared. E_2, genistein, luteolin, enterodiol, coumestrol, ICI 182,780, and *Cyclopia* extract stock solutions were prepared in dimethylsulfoxide (DMSO).

4.2.2. Cell Culture

COS-1, African green monkey kidney fibroblast cells (ATCC, United States of America), and MCF-7BUS human breast cancer cells [46] (a kind gift from A. Soto, Tufts University, Boston, Massachusetts, United States of America) were maintained in high glucose (4.5 g/L) Dulbecco's modified eagle's medium (DMEM) (Sigma-Aldrich®) supplemented with 10% FCS (Highveld Biologicals, South Africa), 100 IU/ml penicillin and 100 µg/ml streptomycin (Gibco, Invitrogen™, South Africa), 2mM glutamine (Merck), 44mM sodium-bicarbonate (Gibco), 1mM sodiumpyruvate

(Gibco), and 0.1mM non-essential amino acids (Gibco). All cells were maintained in a humidified cell incubator, set at 97% relative humidity and 5% CO_2 at 37˚C.

4.2.3. Western Blot

MCF-7BUS cells were seeded into sterile 6-well tissue culture plates at a concentration of 2.5 x 10^5 cells/well and allowed 24 hours to settle. On day two the cells were rinsed once with sterile phosphate buffered saline (PBS) (pre-warmed to 37˚C), medium changed to DMEM without phenol red supplemented with 10% charcoal treated FCS and 1% penicillin and streptomycin mixture, and treated for 24 hours with E_2, polyphenols and *Cyclopia* extracts (concentrations used indicated in figures) where after cells were placed on ice and washed once with ice cold PBS.

COS-1 cells were seeded in sterile 10 cm tissue culture plates at a concentration of 2 x 10^6 cells/plate and allowed 24 hours to settle. On day two the cells were rinsed once with sterile phosphate buffered saline (PBS) (pre-warmed to 37˚C), medium changed to DMEM without phenol red supplemented with 10% charcoal treated FCS and 1% penicillin and streptomycin mixture. Cells were transfected with either ERα (30, 150 or 300 ng hERα/10 cm plate) or ERβ (30, 150, or 300 ng hERβ/10 cm plate) using FuGENE™ 6 transfection reagent (Roche Applied Science, South Africa) as described by the manufacturer. Empty vector (pGL2-Basic from Promega) was used to adjust all transfection conditions to a constant amount of total DNA/condition (300 ng). Human (h) ERα (pSG5-hERα [47]) and ERβ (pSG5-hERβ [48]) expression plasmids were kind gifts from F. Gannon (European Molecular Biology Laboratory, Heidelberg, Germany). Cells were left for 24 hours, replated into sterile 6-well tissue culture plates at a concentration of 2.5 x 10^5 cells/well and allowed 24 hours to settle where after cells were placed on ice and washed once with ice cold PBS.

Cell lysates from washed COS-1 and MCF-7BUS cells were prepared by adding lysis buffer A (10mM Hepes pH 7.5 (Gibco), 1.5mM $MgCl_2$, 10mM KCl, 0.1% NP-40 (Roche Applied Science) and

Complete Mini protease inhibitor cocktail (Roche Applied Science), shaking on ice for 15 min and freezing overnight at -20˚C. On thawing, lysates were transferred to 1.5ml Eppendorf tubes on ice, centrifuged for 10 min at 12 000 x g at 4˚C and the cleared lysates were transferred to 1.5ml Eppendorf tubes on ice, alliquoted and stored at -20˚C until assayed. Lysates (20µl) were separated on a 10% SDS-PAGE gel. Following electrophoresis, proteins were electro-blotted and transferred to a Hybond-ECL nitrocellulose membrane (Amersham Biosciences, South Africa), which was probed for ERα (diluted 1:500), ERβ (1:250) and GAPDH (1:500). Proteins were visualized using HRP labelled anti-rabbit antibody for ERα (1:2500) and ERβ (1:1000), or HRP labelled anti-mouse antibody for GAPDH (1:5000), and ECL Western blotting detection reagents (Pierce®, Thermo Fisher Scientific Inc., U.S.A.) and medical x-ray film (Axim (PTY) LTD., South Africa). All antibodies, primary and secondary, were purchased from Santa Cruz Biotechnology, Inc., U.S.A. To determine relative ERα and ERβ levels the x-ray film bands of ERα, ERβ and GAPDH were quantified using UN-SCAN-IT software and results expressed as the intensity of the ERα or ERβ band relative to the GAPDH band.

4.2.4. Whole-cell binding assays

COS-1 cells transfected with ERα and ERβ for Western blot analysis were also replated for whole-cell binding assays into sterile 24-well tissue culture plates at a concentration of 5 x 10^4 cells/well. Cells were allowed 24 hours to settle and washed three times with 500 µl PBS (pre-heated at 37°C) to remove any endogenous estrogen-like compounds present in the culture medium.

To determine the estrogen receptor subtype affinity (K_d) for E_2, DMEM containing 10 or 20 nM radiolabelled estradiol (^{3}H-E_2, specific activity = 100 Ci/mmol, American Radiolabeled Chemicals, Inc., St. Louis, Missouri, USA) in the absence or presence of increasing amounts of unlabeled E_2 were added to cells, which were incubated at 37 ˚C. After four hours plates were placed on ice, the medium aspirated and the cells washed three times with ice cold bovine serum albumin-PBS (0.2%) for 15

minutes at 4˚C. One hundred microliters of lysis buffer (0.2% (v/v) Triton, 10% (v/v) glycerol, 2.8% (v/v) Tris-phosphate-EDTA and 1.44 mM EDTA) was added to each well where after the plates were shaken at room temperature for 15 min and frozen overnight at -20˚C. On thawing of samples, 5 µl of lysate from each well was used for protein determination using the Bradford method [49]. The remaining lysate was transferred to scintillation vials to which 1.5 ml of scintillation fluid (Quickszint FLOW 2, Zinsser Analytic, South Africa) had been added. Radioactivity of the assay samples was determined using a Perkin Elmer Tri-Carb® B2810TR liquid scintillation analyser and Quantasmart™ software (Separation Scientific, South Africa). As homologous competitive binding was used, the K_d may be directly determined from the IC_{50} value for each concentration of ^3H-E_2 using the following formula: $IC_{50} = [^3H\text{-}E_2] + K_d$. The K_d for the three concentrations of transfected ER of both subtypes was determined by using the global fit model of GraphPad Prism® version 5.10 for Windows (GraphPad Software). A global fit model defines a family of curves and it is useful as it allows sharing of parameters between data sets and for each shared parameter the program will find one (global) best-fit value that is applicable to all data sets. To fit one transfected concentration (30, 150, or 300 ng) of a specific ER subtype data, the program was instructed to fit one value for receptor number (Bmax) and one value for receptor affinity (K_d) that applied to both concentrations of ^3H-E_2 (10 and 20 nM) data sets. Furthermore, to determine the specific estrogen binding for the three concentrations of transfected ER of both subtypes, DMEM containing either 10 nM ^3H-E_2 or 10 nM ^3H-E_2 and a 1000 fold unlabelled E_2 were added to appropriate wells. Cells were incubated at 37 ˚C for four hours where after cells were lysed and the protein concentration and radioactivity determined as described above. Specific binding was calculated as total binding (10 nM ^3H-E_2) – non-specific binding (10 nM ^3H-E_2 + 1000 fold unlabelled E_2). The use of specific binding values is usefull as it corrects for the binding of ^3H-E_2 to any sites other than the ER. Furthermore, the obtained specific binding (cpm/mg protein) and

previously determined K_d values were also used to determine the concentration in fmol ER/mg protein of expressed receptor from the transfected ER using the following equations:

Convert cpm to dpm:

$$dpm = cpm \times (100/\text{Counting Efficiency}) \quad [1]$$

(CE was 37.2% in our system)

Convert dpm to Ci: (1 Ci = 2.22×10^{12} dpm):

$$Ci = dpm \text{ (Equation 1)} / 2.22 \times 10^{12} \quad [2]$$

Convert Ci value to fmol using specific activity (SA) of ligand in Ci/mmol:

SA for ^3H-E_2 = 100 Ci/mmol

$$Fmol\ (10^{-12}\ mol) = ((Ci \text{ (Equation 2)}/SA)) \cdot 10^{12} \quad [3]$$

And thus

$$fmol/mg\ protein = fmol \text{ (Equation 3)} /mg\ protein = Y_{max} \quad [4]$$

The Ymax (Equation 4) in fmol/g protein, Kd, and [Ligand] values in nM are now known and therefore Bmax may be calculated in fmol/mg protein (Equation 5).

$$B_{max} = \frac{(Y_{max}\ ([Ligand] + Kd))}{[Ligand]} \quad [5]$$

4.2.5. Quantification of MCF-7BUS Western Blots

To obtain quantitative ER subtype protein values from the qualitative Western blots the three standard ERα/ERβ concentrations, from the same transfection as used for whole cell binding of which the expressed protein values, in fmol/mg protein, are known, were separated on a 10% SDS-PAGE gel along with the treated MCF-7BUS lysates. ER proteins were visualized using appropriate anti-bodies as described above and the bands were quantified using UN-SCAN-IT software to obtain pixel values,

which represents the ERα or ERβ band intensity relative to the intensity of the GAPDH band. The pixel values of the three standard ERα/ERβ concentrations were plotted against the known ERα/ERβ expressed values in fmol/mg protein to generate a standard curve. Using this standard curve and the pixel values of treated MCF-7BUS samples, quantitative protein values were determined, which was used to determine the ERα:ERβ ratio in treated MCF-7BUS cells.

4.2.6. Live cell nuclear import

COS-1 cells were seeded into eight chamber tissue culture plates on day one at a concentration of 2 x 10^4 cells/chamber and allowed to settle for 24 hours. On day two the cells were rinsed once with sterile phosphate buffered saline (PBS) (pre-warmed to 37˚C) and medium changed to high glucose (4.5 g/L) DMEM without phenol red supplemented with 10% charcoal treated FCS and 1% penicillin and streptomycin mixture. COS-1 cells were transfected with either 200 ng/chamber pCMX-ERα-YFP or 200 ng/chamber pCMX-YFP-ERβ and 600 ng/chamber of the pCMX-pL2 empty vector for a total DNA concentration of 800 ng/chamber using FuGENE™ 6 transfection reagent as described by the manufacturer. The pCMX-ERα-YFP, pCMX-YFP-ERβ and pCMX-pL2 plasmid constructs were kind gifts from Dr. Wei Xu, McArdle Laboratory for Cancer Research, University of Wisconsin [27]. The medium was changed 24 hours after transfection and cells were incubated for an additional 24 hours to allow expression of constructs. Cells were analysed at the Stellenbosch University's central analytical facility imaging unit in the temperature-controlled chamber (37 ˚C) of an Olympus Cell system attached to an IX-81 inverted fluorescence microscope equipped with an F-view-II cooled CCD camera and a 150W Xenon lamp as light source, which is part of the MT20 excitation source. An Olympus Plan Apo N 60X/1.4 oil objective, an YFP filter set and the Cell® imaging software were used for image acquisition and analysis. Cells were induced with E_2, polyphenols and *Cyclopia* extracts (concentrations used indicated in figures) and YFP images were taken every minute over a 30 min

period. Nuclear import was quantified as the increase in YFP fluorescence in the nucleus (within a region of interest) over the period of stimulation. Fluorescence in the nucleus of solvent treated control cells was subtracted from all time points (baseline correction) and a one phase exponential association curve was fit to the data. The generated half time ($t_{1/2}$) represents the time it takes to achieve 50% of maximal YFP nuclear accumulation.

To determine nuclear YFP-ER distribution cells with clear nuclear distribution was chosen at time points 0, 15, and 30 minutes. As long a line as possible was drawn through the nuclei of selected cells avoiding nucleoli and the Cell® imaging software was used to quantify YFP fluorescence intensity along this line. GraphPad Prism® software was used to quantify the coefficient of variation (CV) of YFP fluorescence intensity along the drawn line. A lower CV indicates a more random nuclear distribution, while a higher CV value is indicative of a more ordered nuclear distribution [50].

4.2.7. Data manipulation and statistical analysis

The GraphPad Prism® version 5.10 for Windows (GraphPad Software) was used for graphical representations and statistical analysis. One-way ANOVA and Dunnett's post-test comparing all columns to the solvent control were used for statistical analysis and significance is displayed on the graphs. For all experiments the error bars represent the SEM of at least two independent experimental repeats.

4.3. Results

4.3.1. The Cyclopia extracts down-regulated ERα, while up-regulating ERβ in the human breast cancer cell line, MCF-7BUS

Estrogens exert their biological function through the ER, which exists as two subtypes, ERα and ERβ [2,6-8].

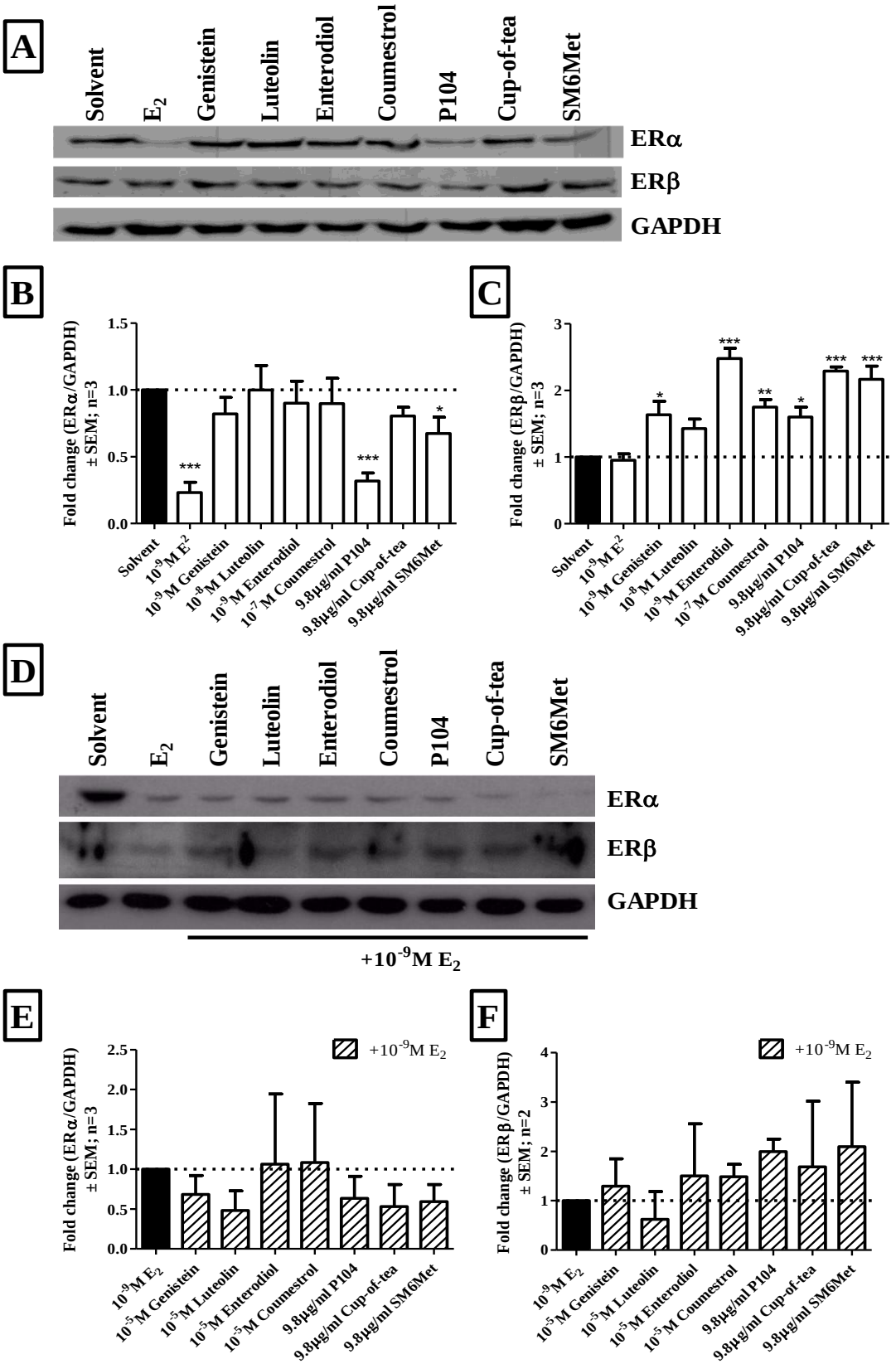


Figure 1. In MCF-7BUS cells the *Cyclopia* extracts down-regulated ERα protein levels and up-regulated ERβ levels. Clear lysates of MCF-7BUS cells treated with either E_2, polyphenols, or *Cyclopia* extracts in the absence (A, B & C) or presence (D, E & F) of 10^{-9}M E_2 were subjected to Western blotting and probed with subtype specific ERα- (A, B, D & E) and ERβ (A, C, D & F) antibodies. (A & D) Representative blots of independent experiments that were quantified with UNSCAN-IT software (B, C, E & F). Statistical analysis was done using One-way ANOVA with Dunnett's post-test comparing all columns to the either solvent control (B & C) or 10^{-9}M E_2 (E & F) (*, P<0.05; **, P<0.01; ***, P<0.001). The dotted line through the bars represents the values for solvent control (B & C) or 10^{-9}M E_2 (E & F). Mean ± SEM is of three (B, C & E) or two (F) independent experiments.

ERα is associated with cell proliferation and the occurrence of breast cancer, while several studies have shown that ERβ acts as an antagonist of ERα in breast cancer and could act as an inhibitor of breast cancer development [20-26]. Furthermore, higher ERα levels are associated with malignant tumours, while higher ERβ levels are associated with benign tumours [25,30]. Therefore, we evaluated the effect of *Cyclopia* extracts on the ER subtype levels in MCF-7BUS, which express both subtypes (Chapter 3).

Western blots indicate that ERα protein levels were significantly down-regulated (by 77%) by 10^{-9}M E_2 when compared to solvent treated cells (Figs. 1A&B). The methanol extracts of *Cyclopia*, P104 and SM6Met, like E_2, also significantly down-regulated ERα protein levels, by 68% and 33%, respectively of solvent values. The water extract, cup-of-tea, also down-regulated ERα protein levels (by 20%), however, values did not reach significance. In contrast, the polyphenols had no significant effect on ERα protein levels. E_2 had no significant effect on ERβ protein levels (5% down-regulation) unlike all of the *Cyclopia* extracts, which significantly up-regulated ERβ protein levels (P104 by 160%, cup-of-tea by 229%, and SM6Met by 217%) (Figs. 1A&C). All of the polyphenols, like the *Cyclopia* extracts, up-regulated ERβ protein levels although the luteolin values did not reach significance.

We also evaluated the effect of *Cyclopia* extracts on ERα and ERβ protein levels in the presence of 10^{-9}M E_2 (concentration reflects pre-menopausal E_2 levels [51] and was found to induce highest MCF-7BUS cell proliferation in Chapter 3). All of the extracts of *Cyclopia*, in the presence of 10^{-9}M E_2 were

able to down-regulate, although not significantly, ERα levels further than E_2 alone (P104 by 37%, cup-of-tea by 47%, and SM6Met by 41% relative to E_2 alone) (Figs. 1D&E), which correlates with the findings that *Cyclopia* extracts on their own possessed the ability to down-regulate ERα (Fig. 1B). The polyphenols, genistein and luteolin, like the *Cyclopia* extracts, also down-regulated ERα protein levels in the presence of 10^{-9}M E_2. However, ERβ levels in the presence of E_2 were up-regulated by all of the *Cyclopia* extracts and most of the polyphenols, except luteolin, although the values did not reach significance (Figs. 1 D&F).

In conclusion, the *Cyclopia* extracts down-regulated ERα protein levels in MCF-7BUS cells, both in the absence (Fig. 1B) and presence (Fig. 1E) of E_2, while all of the *Cyclopia* extracts stabilized ERβ protein levels in both the absence (Fig. 1C) and presence (Fig. 1F) of E_2. Considering the known roles of the ER subtypes in breast cancer [20-26] these results may be seen as positive attributes of the *Cyclopia* extracts in terms of breast cancer prevention or treatment. However, as several studies have suggested that the ERα:ERβ ratio rather than the absolute subtype levels may be important in breast cancer [31-33] we next set out to evaluate the effects of the *Cyclopia* extracts on this ratio.

4.3.2. Combining Western blotting with whole cell binding allows for the quantification of ER subtype protein levels.

Western blotting is a technique most suited to evaluating relative changes in concentrations of specific proteins like ERα and ERβ, however, as ERα and ERβ specific antibodies may have different affinities for their cognate proteins, Western blotting alone would not be adequate to evaluate the modulation of the ERα:ERβ ratio. To overcome this we reassessed results obtained with Western blotting, which could illustrate subtype specific modulation of ER levels by treatments, using a standard curve where pixels obtained from Western blots are correlated with ER concentration in fmol/mg protein as determined with whole cell binding using radiolabelled E_2 (Fig. 2).

First, towards determining expressed ER concentration in fmol/mg protein, we determined the K_d (binding affinity) values for all three concentrations (30, 150, and 300ng) of transfected ERα (Fig. 3A) and ERβ (Fig. 3B) in COS-1 cells.

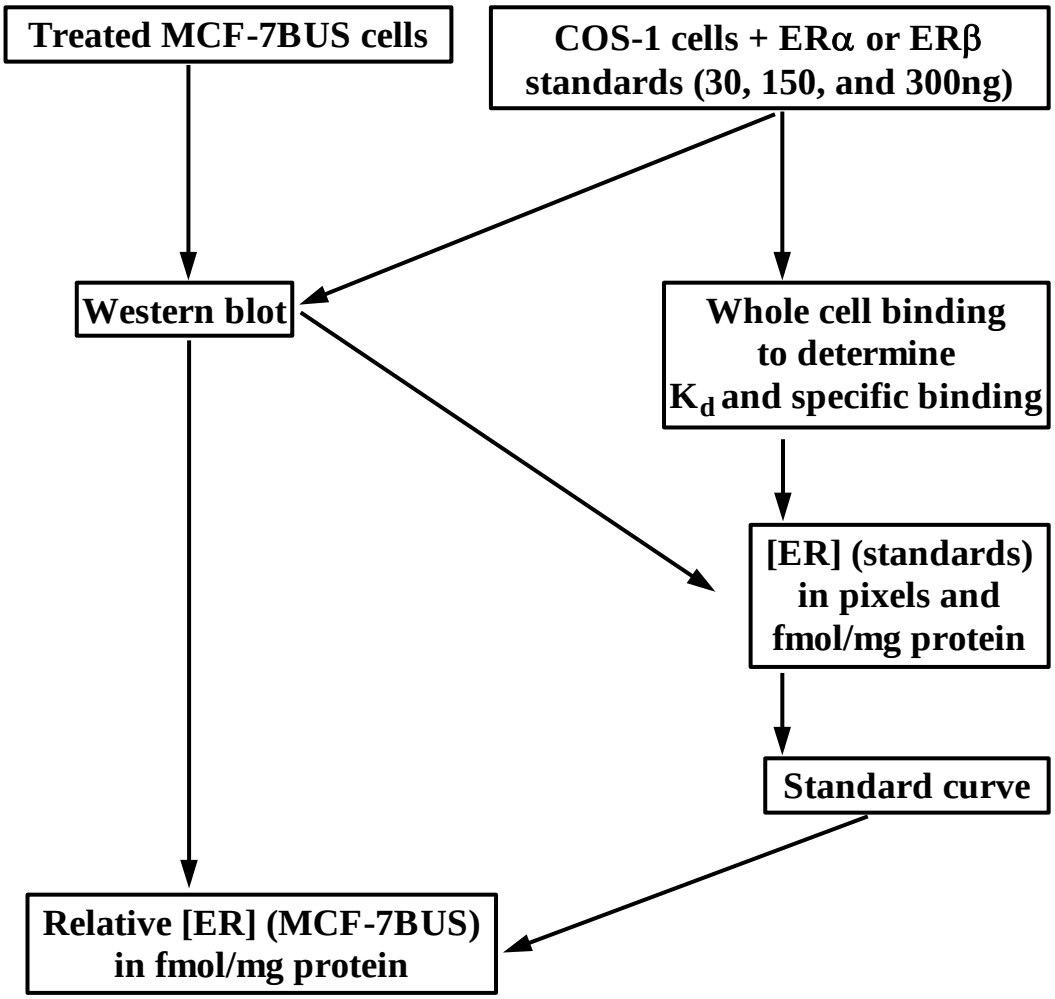


Figure 2. The sequence of experimental procedures to quantify relative ERα and ERβ protein levels in MCF-7BUS cells after treatment. COS-1 cells were transiently transfected with 30, 150, and 300 ng ERα or ERβ. The transfected cell were subjected to Western blotting as well as whole cell binding to determine the ER subtype concentrations in both pixels and fmol/mg protein. From these values a standard curve was generated, which was used to determine relative ER subtype concentrations in MCF-7BUS cells treated with polyphenol and *Cyclopia* extracts in the absence or presence of 10^{-9}M E_2.

The K_d value was determined for each concentration of ER subtype, as it has been shown that increasing concentrations of steroid receptor results in co-operative ligand binding, which may influence the binding affinity of the ligand [52-54].

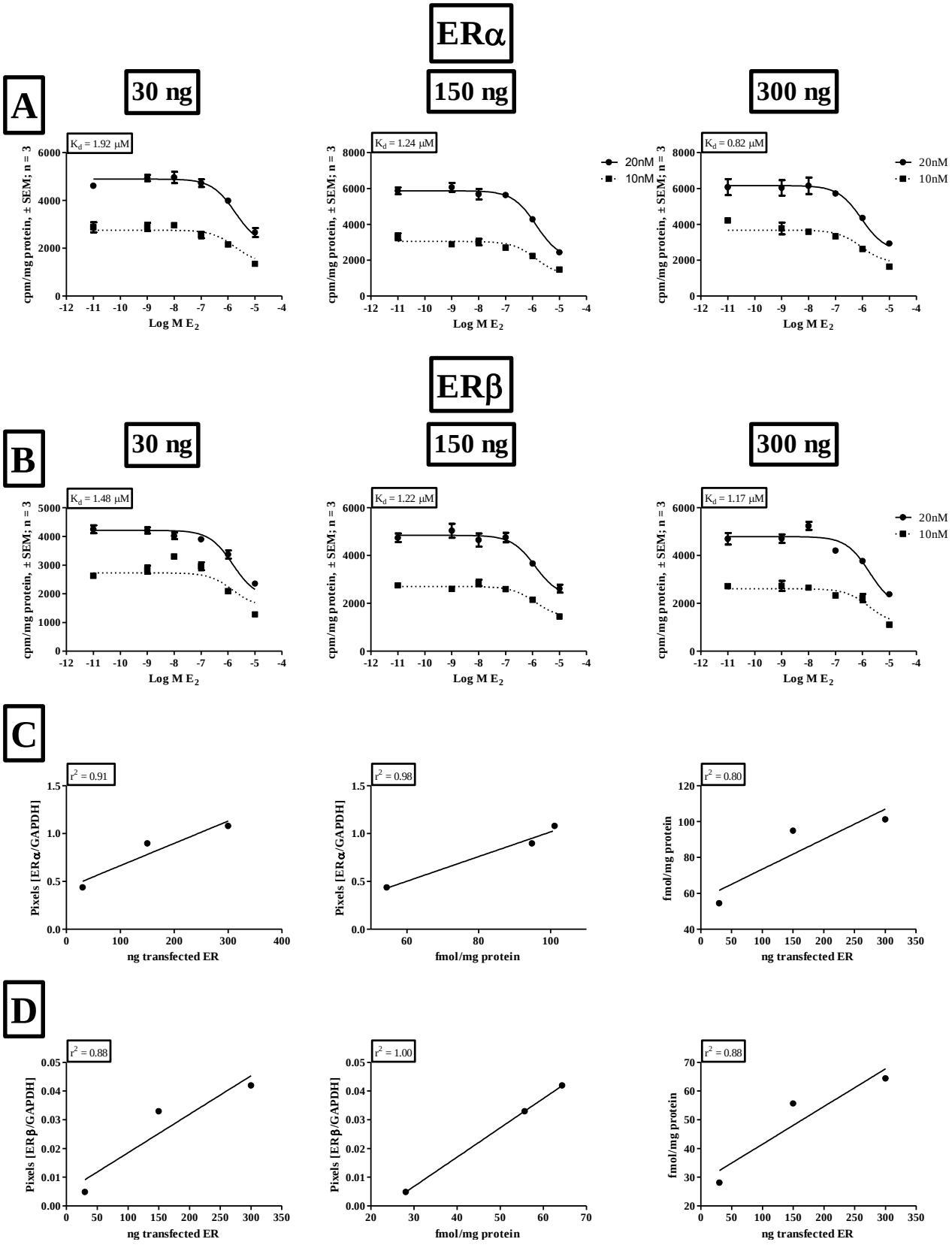


Figure 3. Combining Western blotting with whole cell binding for the quantitative quantification of ER subtype protein levels. The binding affinity of E_2 for (A) ERα and (B) ERβ at 30, 150, and 300 ng transfected ER was determined using competitive whole cell binding. Clear lysates of treated MCF-7BUS and untreated COS-1 cells (COS-1 cells transfected with either 30ng, 150ng, or 300ng pSG5-hERα or 30ng, 150ng, and 300ng pSG5-hERβ) were subjected to Western blotting and probed with subtype specific ERα- and ERβ antibodies, quantified with UNSCAN-IT software, and standard curves were generated plotting relative (C) pSG5-hERα or (D) pSG5-hERβ protein band intensities, in pixel concentration, against either ng transfected ER or fmol expressed ER/mg protein. Expressed ER protein levels (fmol ER/mg protein) were calculated using experimentally determined K_d values as well as a published K_d values [55]. Mean ± SEM is of two to three independent experiments. Figures (C) and (D) are representative standard curves of 6 independent experiments as standard curves are generated after each Western blot.

Our K_d values indeed decreased, indicating increased affinity, within increasing concentrations of transfected ER, which supports the findings that higher ER concentrations display co-operative ligand binding. However, our K_d values were in the μM range whereas most published K_d values for ER are in the nM range [52,54-56]. Although unlikely, the difference between the experimentally determined Kd values and the published Kd values may be ascribed to the presence of tritiated E_2 binders other than the ER, which, in future, should be evaluated by performing whole cell binding studies in untransfected COS-1 cells. To evaluate the influence of this difference we used both our experimentally determined K_d values as well as published K_d values (ERα = 0.05 nM and ERβ = 0.09 nM) [55]) to calculate the concentration of ERα and ERβ. As Table 1 shows the difference in K_d values had little effect on the calculation of ER concentration in fmol/mg protein.

Having established the K_d values for each concentration of transfected ER we determined the concentration of expressed ER in fmol/mg protein using the equations described in the materials and methods section of this chapter. Western blots were performed on lysates of COS-1 cells transfected with the three standard ER concentrations used to determine the K_d values. Linear standard curves were generated by plotting the band intensities, in pixels, of the Western blots obtained with the three ER standards against the ng transfected ER, as well as the calculated fmol expressed ER/mg protein for ERα (Fig. 3C) and ERβ (Fig. 3D). Furthermore, we also plotted ng transfected ER against fmol

expressed ER/mg protein. The correlation between pixels and fmol expressed ER/mg protein was excellent (r^2 varying between 0.98 and 1.00), however, the correlation between pixels and ng transfected ER and ng transfected ER and fmol expressed ER/mg protein was less good (r^2 varying between 0.80 and 0.91). This may be due to the fmol/mg protein values not linearly increasing as more ER is transfected into the COS-1 cells and therefore, the doubling of ng transfected ER (150ng to 300ng) is not reflected by a doubling of fmol/mg protein expressed. We would thus expect that calculation of the ERα:ERβ ratio using ng transfected ER would be higher than the ratio calculated using fmol expressed ER/mg protein. We used both ng transfected ER as well as fmol expressed ER/mg protein to calculate the ERα:ERβ ratios, however, as the correlation between pixels and fmol expressed ER/mg protein is higher we would accord more weight to ERα:ERβ ratios calculated in this manner.

4.3.3. Cyclopia extracts decreased the ERα:ERβ ratio in MCF-7BUS cells.

The co-expression of ERα and ERβ has been shown to inhibit the proliferative effect of ERα [20-26]. Also, cells expressing higher levels of ERα compared to ERβ show ERα dependent increases in growth while growth is inhibited in cells expressing equal amounts of ERα and ERβ. Therefore, it has been suggested that the ratio of ERα:ERβ in the same tumour cell is an important to predictor of the physiological effect of treatments [32,57,58]. We thus propose that treatments that increase the relative amount of ERβ compared to ERα would be favourable for breast cancer treatment and/or prevention and wanted to evaluate the modulation of the ERα:ERβ ratio in MCF-7BUS cells treated with *Cyclopia* extracts.

Western blots were performed on lysates from treated MCF-7BUS cells together with lysate standards prepared in COS-1 cell for which fmol expressed ER/mg protein had been established using whole-cell binding (Fig. 2). Standard curves were established for each ER subtype (Fig. 3C & D) and used to

correlate band intensity (pixels) of lysates from the treated MCF-7BUS cells with ng transfected ER as well as fmol expressed ER/mg protein.

Table 1. The modulation of relative ERα:ERβ levels by polyphenols and *Cyclopia* extracts in both the absence and presence of E_2 in MCF-7BUS cells. MCF-7BUS cells were treated with polyphenols and *Cyclopia* extracts in the absence (A) or presence (B) of 10^{-9}M E_2. Standard curves, generated as explained in the materials and methods section, were used determine relative ERα:ERβ levels after 24 hour treatment period. Statistical analysis was done using One-way ANOVA with Dunnett's post-test comparing all columns to the solvent treated (A) ($^{\#}$, $p<0.05$; $^{\#\#}$, $p<0.01$; $^{\#\#\#}$, $p<0.001$) or 10^{-9}M E_2 (A&B) treated cells (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$). Mean ± SEM is of two to three independent experiments.

A

	ERα : ERβ Ratio (ng Transfected receptor)	ERα : ERβ Ratio (fmol/mg protein Experimental K_d)	ERα : ERβ Ratio (fmol/mg protein Literature K_d)
Solvent	1 : 4.02 ± 0.01***	1 : 3.18 ± 0.74***	1 : 3.32 ± 0.88***
10^{-9}M E_2	1 : 59.71 ± 4.70 $^{\#\#\#}$	1 : 27.59 ± 2.47 $^{\#\#\#}$	1 : 28.91 ± 1.16 $^{\#\#\#}$
10^{-9}M Genistein	1 : 18.28 ± 1.51**	1 : 8.43 ± 0.61**	1 : 8.90 ± 1.08**
10^{-9}M Luteolin	1 : 18.45 ± 5.01**	1 : 5.19 ± 0.95***	1 : 5.50 ± 1.29***
10^{-9}M Enterodiol	1 : 23.65 ± 9.36**	1 : 8.49 ± 3.40***	1 : 9.01 ± 3.92**
10^{-9}M Coumestrol	1 : 25.19 ± 6.59**	1 : 8.52 ± 3.51***	1 : 9.02 ± 3.96**
9.8µg/ml P104	1 : 32.86 ± 2.45* $^{,\#}$	1 : 15.18 ± 1.29* $^{,\#}$	1 : 15.91 ± 0.57* $^{,\#}$
9.8µg/ml Cup of Tea	1 : 46.81 ± 0.72 $^{\#\#}$	1 : 21.61 ± 0.10 $^{,\#\#}$	1 : 22.75 ± 1.23 $^{,\#\#}$
9.8µg/ml SM6Met	1 : 24.82 ± 8.01**	1 : 6.27 ± 1.58***	1 : 6.25 ± 1.60***

B

	ERα : ERβ Ratio (ng Transfected receptor)	ERα : ERβ Ratio (fmol/mg protein Experimental K_d)	ERα : ERβ Ratio (fmol/mg protein Literature K_d)
10^{-9}M E_2	1 : 49.59 ± 10.48	1 : 24.11 ± 3.76	1 : 24.96 ± 4.01
+ 10^{-9}M Genistein	1 : 33.11 ± 23.30	1 : 19.05 ± 13.90	1 : 18.95 ± 18.83
+ 10^{-9}M Luteolin	1 : 9.59 ± 6.36	1 : 5.12 ± 3.24	1 : 5.10 ± 3.22
+ 10^{-9}M Enterodiol	1 : 124.50 ± 122.60	1 : 72.65 ± 71.68	1 : 72.28 ± 71.32
+ 10^{-9}M Coumestrol	1 : 45.13 ± 39.44	1 : 26.19 ± 23.21	1 : 26.06 ± 23.09
+ 9.8µg/ml P104	1 : 45.66 ± 20.80	1 : 25.93 ± 12.90	1 : 25.80 ± 12.83
+ 9.8µg/ml Cup of Tea	1 : 47.16 ± 35.47	1 : 27.20 ± 21.07	1 : 27.06 ± 20.96
+ 9.8µg/ml SM6Met	1 : 59.55 ± 38.22	1 : 34.15 ± 22.97	1 : 33.98 ± 22.85

We used not only the experimentally calculated K_d values but also literature K_d values to determine fmol expressed ER/mg protein. Generally, as expected, the ERα:ERβ ratio calculated using fmol expressed ER/mg protein was higher than when calculated using ng transfected ER, however no significant difference was observed between ratios of fmol expressed ER/mg protein calculated using experimental vs. literature K_d values (Table 1). Treatment with 10^{-9}M E_2 significantly increased the amount of ERβ relative to ERα (Table 1A) and this may be attributed to the robust down-regulation of ERα protein levels by E_2 without any significant effect on ERβ protein levels (Figs. 1A, B, & C). The *Cyclopia* extracts, P104 and cup-of tea, also significantly up-regulated the levels of ERβ relative to ERα when compared to solvent treated cells, but not to the same extent as E_2. Furthermore, this elevated ratio of ERα:ERβ was obtained by down-regulating ERα and up-regulating ERβ, while E_2 only down-regulated ERα (Figs. 1A, B, & C). SM6Met had no significant effect on the ERα:ERβ ratio when compared to solvent, although it was able to both down-regulate ERα protein levels and up-regulate ERβ protein levels (Figs. 1A, B, & C).

The same experimental approach was followed to evaluate the changes in the ERα:ERβ ratio exerted by treatment with the *Cyclopia* extracts in the presence of 10^{-9}M E_2 (Table 1B). Although no significant changes in the ERα:ERβ ratio relative to E_2 was observed, a trend towards higher ERβ relative to ERα protein levels is observed with the SM6Met extract and E_2 co-treatment compared to E_2 treatment alone. Furthermore, when comparing effects of *Cyclopia* extracts alone with effects in the presence of E_2 it is clear that E_2 further reduces the ERα:ERβ ratio.

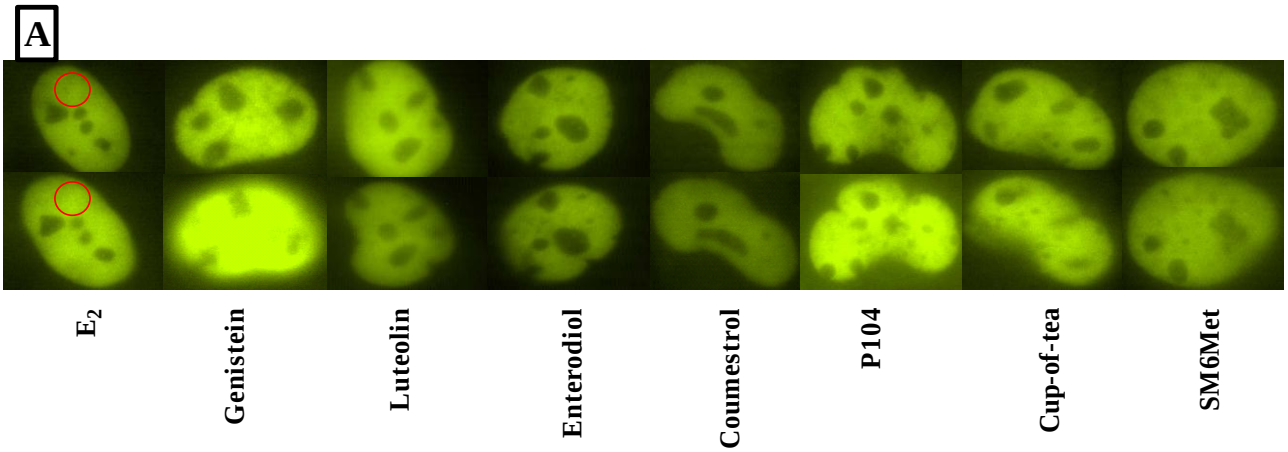
To conclude, our findings suggest that although the phytoestrogenic *Cyclopia* extracts modulated the ERα:ERβ ratio in the same manner as E_2 (Table 1), the mechanism whereby the ratio was modulated is different from that of E_2 in that E_2 only down-regulated ERα, while, the *Cyclopia* extracts both down-regulated ERα and up-regulated ERβ protein levels (Fig. 1).

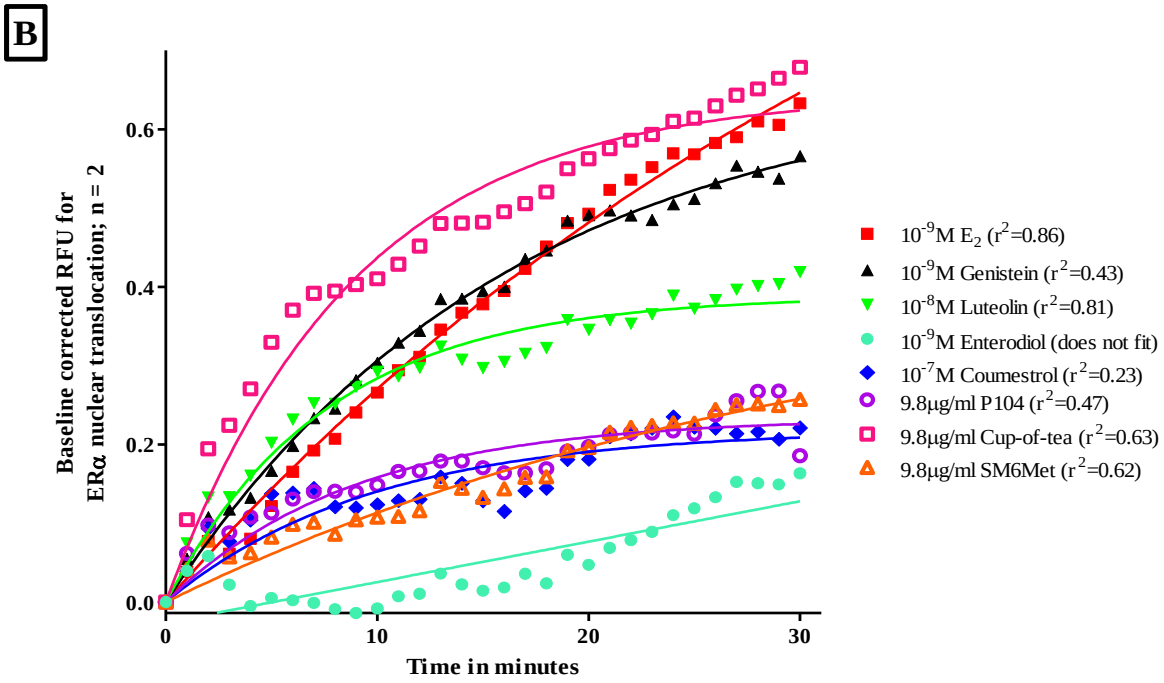
4.3.4. The methanolic extracts of Cyclopia induced increased nuclear localization of ERβ, but reduced nuclear localization of ERα, when compared to E_2.

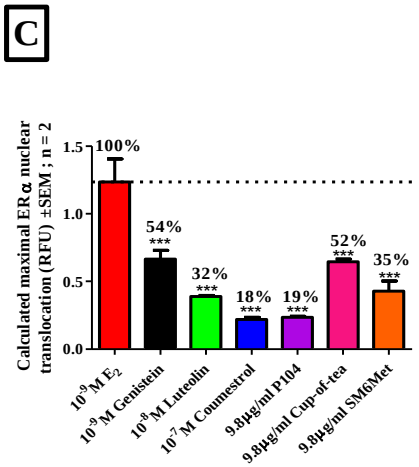
Although the ER resides mostly in the nucleus of cells, the receptor does move between the nucleus and the cytoplasm [13-15]. Furthermore, findings that the pure ER antagonists, fulvestrant and ICI 164,384, disrupt movement of the ER into the nucleus by either blocking re-uptake of the ER into the nucleus or by concentrating the ER in other cellular compartments, thereby contributing to the disruption of ER signalling [13], prompted us to evaluate how treatment with *Cyclopia* extracts would alter nuclear import rate and maximum nuclear localization of both ER subtypes. COS-1 cells transfected with either yellow fluorescent protein tagged ERα (YFP-ERα) or YFP-ERβ were used to monitor nuclear translocation in real time in live cells.

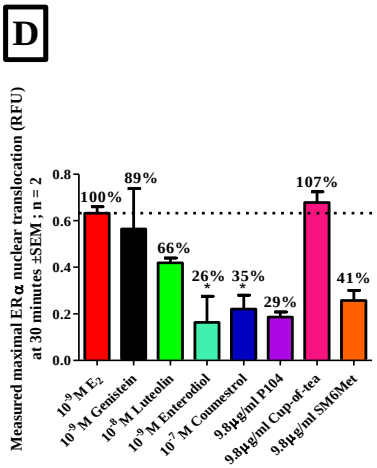
The YFP-ERα (Fig. 4A) as well as the YFP-ERβ (Fig. 5A) resided mostly in the nucleus of cells at 0 minutes of treatment (Figs. 4A & 5A, top panel). By selecting a region of interest (ROI) within each nucleus, shown in red in Figs. 4A & 5A for E_2 treatment as an example, we could measure the change in fluorescent intensity between 0 and 30 minutes (Figs. 4A & 5A, bottom panel) of treatment. Changes in fluorescence intensity are indicative of changes in ER number and the changes between 0 and 30 minutes are shown for ERα (Fig. 4B) and ERβ (Fig. 5B). The nuclear localization may be plotted using exponential one phase association curves to determine maximal nuclear localization and the rate of nuclear localization as the time required to reach 50% of maximal nuclear localization ($t_{1/2}$).

For ERα (Fig. 4B&C), the calculated maximal nuclear localization of YFP-ERα after 30 minutes of treatment with 10^{-9}M E_2 was set as 100% import (Fig. 4C). The *Cyclopia* extracts resulted in significantly lower localization of ERα (19%, 52%, and 35% for P104, cup-of-tea, and SM6Met, respectively).









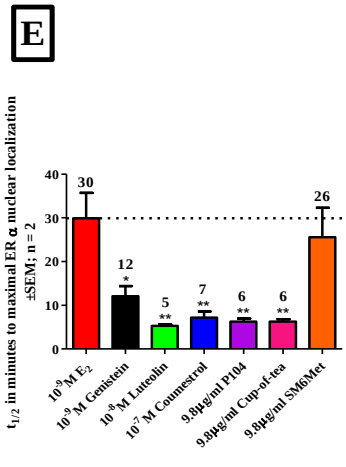


Figure 4. Treatment for 30 minutes with *Cyclopia* extracts induces faster nuclear import of ERα, compared to E_2, but does not concentrate ERα in the nucleus to the same extent as E_2. COS-1 cell were transiently transfected with (A) YFP-ERα and fluorescent intensity was monitored for 30 minutes (A & B). (A) Represents single cells treated with 10^{-9}M E_2, 10^{-9}M genistein, 10^{-8}M luteolin, 10^{-9}M enterodiol, 10^{-7}M coumestrol, 9.8µg/ml P104, 9.8µg/ml cup-of-tea, or 9.8µg/ml SM6Met. YFP images were taken every 10 seconds for 30 minutes and (A) is representative of a single cell at time point 0 (top panel) and 30 (bottom panel) minutes. The red circle within the nucleus represents the region of interest (ROI). (B) Representative graph depicting changes in fluorescent intensity, within the ROI, over 30 minutes. Values for solvent control were baseline subtracted. Representative graphs depicting calculated maximal nuclear localization (C), measured maximal nuclear localization (D) and $t_{1/2}$ values (E) for this period. The calculated maximal nuclear localization and $t_{1/2}$ values were determined with the GraphPad Prism® software fitting a one-phase association curve. Statistical analysis was done using One-way ANOVA with Dunnett's post-test comparing all columns to the E_2 treated cells (*, P<0.05; **, P<0.01; ***, P<0.001). Mean ± SEM is of two cells/treatment of two independent experiments. r^2 value depicts goodness of fit. (C-E) Dotted line through graphs represents 10^{-9}M E_2 values.

However, these calculated values were obtained using an exponential one-phase association curve fit and therefore, the accuracy of the obtained maximal nuclear localization values are dependent on the quality of curve fit as well as the plateauing of the curve indicating that an equilibrium between import and export has been obtained. As the quality of the fit, depicted by r^2 values in the figure legend (Fig. 4B), was not good (r^2 values should be as close to 1 as possible) and no distinguishable plateau was reached after 30 minutes for several treatments, the calculated theoretical maximal nuclear localization values do not always reflect what is observed in the graph. Therefore, the measured values at the 30 minute time point were also plotted for each of the treatments (Fig 4D). At 30 minutes, the measured ERα nuclear localization values after treatment with the methanol extracts of *Cyclopia* still resulted in low ERα nuclear localization in comparison to E_2, reflecting the calculated maximum localization values. However, treatment with the water extract, cup-of-tea, in contrast to the calculated findings, had a similar measured ERα nuclear localization value as E_2. Treatment with the polyphenols, like with the *Cyclopia* extracts, resulted in significantly lower calculated ERα nuclear localization when compared to E_2 treatment. Calculated values for enterodiol, however, could not be obtained as the exponential one-phase association curve could not be accurately fitted. Calculated maximal localization values of ERα for the polyphenols generally held when measured at 30 minutes with the exception of genistein and luteolin, which displayed increased nuclear localization relative to E_2 when measured at 30 minutes.

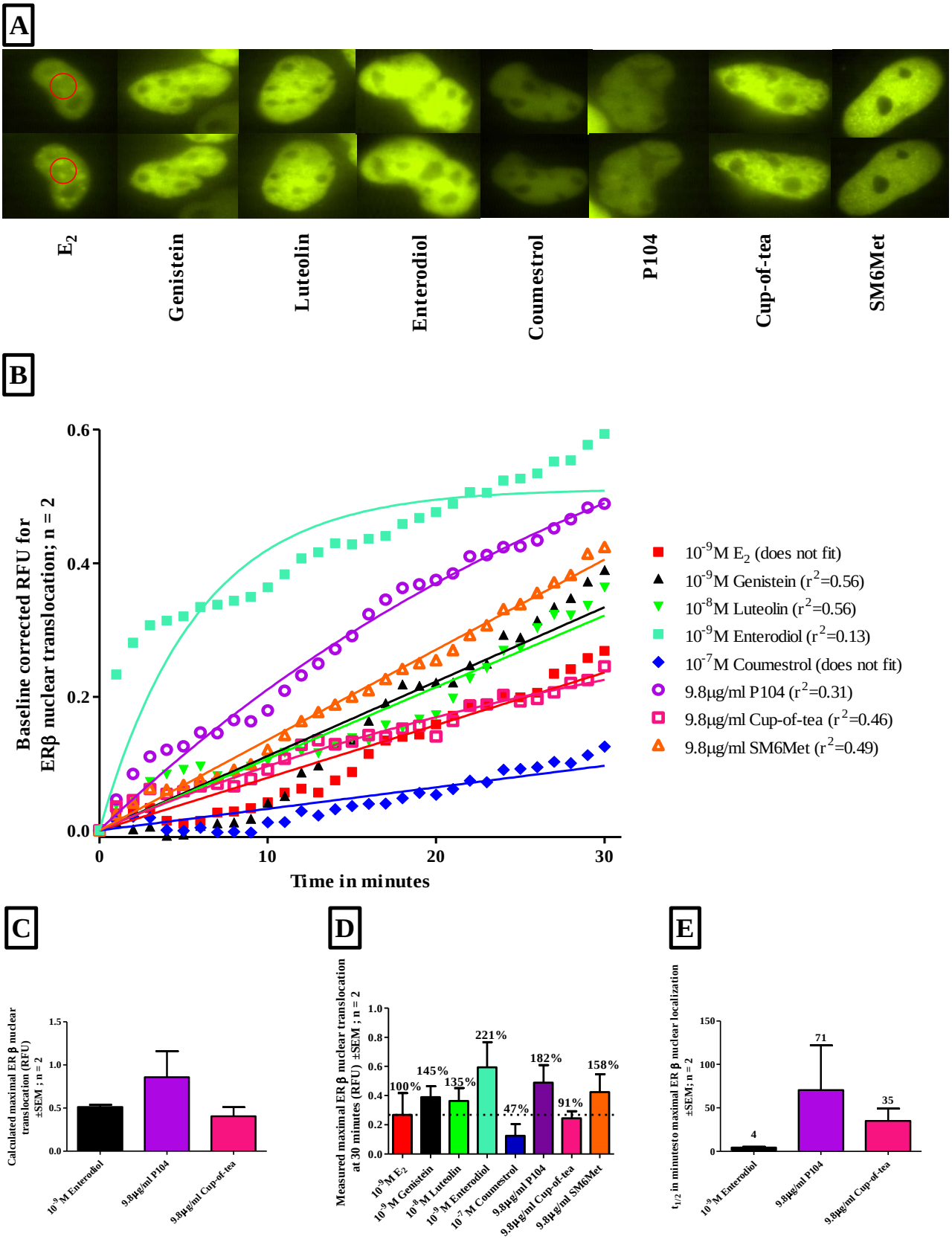


Figure 5. During a 30 minute period *Cyclopia* extracts, to a larger extent than E_2, concentrates ERβ in the nucleus, but the rate of ERβ import into the nucleus is slower than that of E_2. COS-1 cell were transiently transfected with YFP-ERβ and fluorescent intensity was monitored for 30 minutes (A & B). (A) Represents single cells treated with 10^{-9}M E_2, 10^{-9}M genistein, 10^{-8}M luteolin, 10^{-9}M enterodiol, 10^{-7}M coumestrol, 9.8µg/ml P104, 9.8µg/ml cup-of-tea, or 9.8µg/ml SM6Met. YFP images were taken every 10 seconds for 30 minutes and (A) is representative of a single cell at time point 0 (top panel) and 30 (bottom panel) minutes. The red circle within the nucleus represents the region of interest (ROI). (B) Representative graph depicting changes in fluorescent intensity, within the ROI, over 30 minutes. Values for solvent control were baseline subtracted. Representative graphs depicting calculated maximal nuclear localization (C), measured maximal nuclear localization (D) and $t_{1/2}$ values (E) for this period. The calculated maximal nuclear localization and $t_{1/2}$ values were determined with the GraphPad Prism® software fitting a one-phase association curve. Statistical analysis was done using One-way ANOVA with Dunnett's post-test comparing all columns to the E_2 treated cells (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$). Mean ± SEM is of two cells/treatment of two independent experiments. r^2 value depicts goodness of fit. (C-E) Dotted line through graphs represents 10^{-9}M E_2 values.

Exponential one-phase association curves, however, are useful as they allow us to determine $t_{1/2}$ values (time to 50% maximal localization), but, as these values are dependent on the graph reaching a plateau (maximal localization) it could not be determined for treatments, like enterodiol, for which no plateau was reached. The $t_{1/2}$ values for ERα nuclear localization of the polyphenols and the *Cyclopia* extracts were significantly lower than that of E_2, except for the SM6Met extract which was very similar to that of E_2 (25.58 ± 6.75 vs. 29.94 ± 5.78 minutes, respectively) (Fig. 4E).

With regards to ERβ (Fig. 5B), a plateau was not reached for most treatments after 30 minutes and thus maximum nuclear import values could either not be calculated or gave unrealistic values. The exceptions were the polyphenol, enterodiol, and the *Cyclopia* extracts, P104 and cup-of-tea (Fig. 5C). Therefore $t_{1/2}$ values could only be calculated for enterodiol, P104 and cup-of-tea. Although we are therefore not able to compare $t_{1/2}$ values with that of E_2 we can compare the $t_{1/2}$ values for ERβ of these compounds with that of the $t_{1/2}$ values for ERα. With regards to P104 and cup-of-tea, the $t_{1/2}$ values for ERβ were higher than those obtained for ERα (70.46 ± 51.52 vs. 6.27 ± 0.65 and 34.94 ± 14.31 vs. 6.24 ± 0.58 minutes, respectively). Furthermore, with regards to the measured maximum nuclear localization values at 30 minutes, in contrast to ERα (Fig. 4), treatment with all of the polyphenols, except for coumestrol, and with the methanol extracts of *Cyclopia* resulted in higher measured maximal ERβ nuclear localization than with E_2.

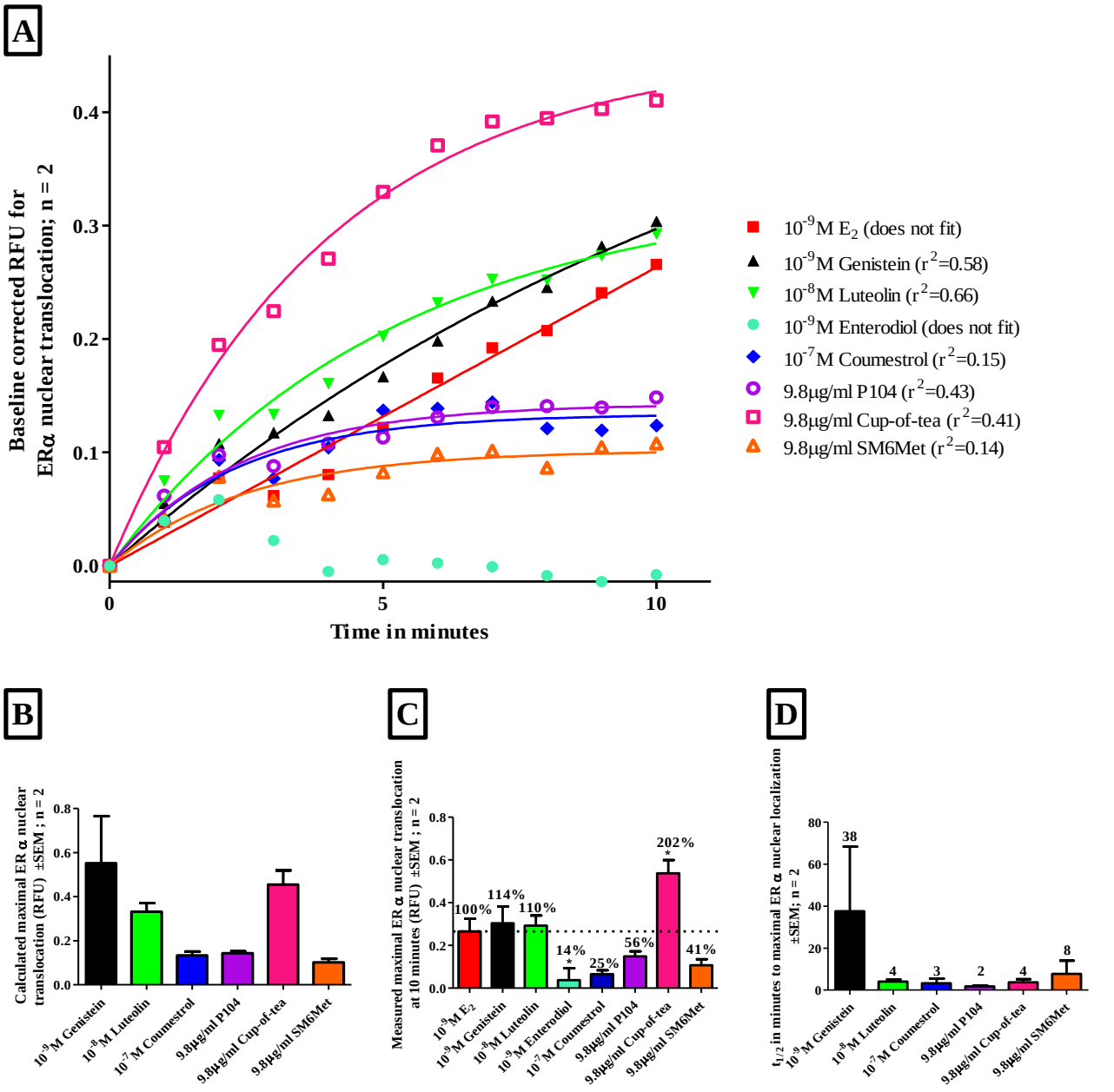


Figure 6. Treatment for 10 minutes with *Cyclopia* extracts induces faster nuclear import of ERα, compared to E_2, but the methanol extracts of *Cyclopia* does not concentrate ERα in the nucleus to the same extent as E_2. COS-1 cell were transiently transfected with YFP-ERα and fluorescent intensity was monitored for 10 minutes with YFP images taken every 10 seconds. (A) Representative graph depicting changes in fluorescent intensity, within the ROI, over 10 minutes. Values for solvent control were baseline subtracted. Representative graphs depicting calculated maximal nuclear localization (B), measured maximal nuclear localization (C) and $t_{1/2}$ values for this period (D). The calculated maximal nuclear localization and $t_{1/2}$ values were determined with the GraphPad Prism® software fitting a one-phase association curve. Statistical analysis was done using One-way ANOVA with Dunnett's post-test comparing all columns to the E_2 treated cells (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$). Mean ± SEM is of two cells/treatment of two independent experiments. r^2 value depicts goodness of fit. (B-D) Dotted line through graphs represents 10^{-9}M E_2 values.

Upon closer inspection of the data obtained with the 30 minutes treatment of cells transfected with YFP-ERα (Fig. 4B) and YFP-ERβ (Fig. 5B) we observed an initial plateau of fluorescence intensity values before a second increase in fluorescence intensity. This is most obvious for cup-of-tea via ERα (Fig. 4) and enterodiol and P104 via ERβ (Fig. 5). To investigate this, we generated graphs using only the data points obtained within the first 10 minutes of treatment and determined the maximal localization (calculated and measured) and $t_{1/2}$ values for both ERα (Fig. 6) and ERβ (Fig. 7) for this shorter time period. Although generally the fit of the curves were not as good as for the 30 minute period we used the data to support conclusions reached concerning the *Cyclopia* extracts using the 30 minute data sets.

For ERα, measured maximum nuclear localization at 10 minutes confirmed (Fig. 6C) the fact that the methanol extracts of *Cyclopia* resulted in a lower maximal nuclear localization, while the water extract, cup-of-tea, resulted in a maximal nuclear localization either similar (30 minutes) or higher (10 minutes) than that achieved by E_2. Similarly, the absolute $t_{1/2}$ values at 10 minutes suggest that the low values obtained at 30 minutes for P104 and cup-of-tea hold. Although the $t_{1/2}$ values for SM6Met appeared very different at the different time points (26 vs. 8 minutes), if we calculated the measured $t_{1/2}$ values as a percentage of the total time (10 or 30 minutes) the proportional $t_{1/2}$ values for the initial 10 minutes correlate well with the 30 minute values for all of the *Cyclopia* extracts. For example, at 10 minutes SM6Met has a calculated t1/2 value of 8 minutes but a proportional t1/2 of 80% (8/10), while at 30 minutes SM6Met has a calculated t1/2 value of 26 minutes but a proportional t1/2 of 86% (26/30). Similarly, the proportional t1/2 values for P104 are 20% at 30 minutes and 20% at10 minutes and for cup-of-tea they are 20% at 30 minutes and 40% at 10 minutes.

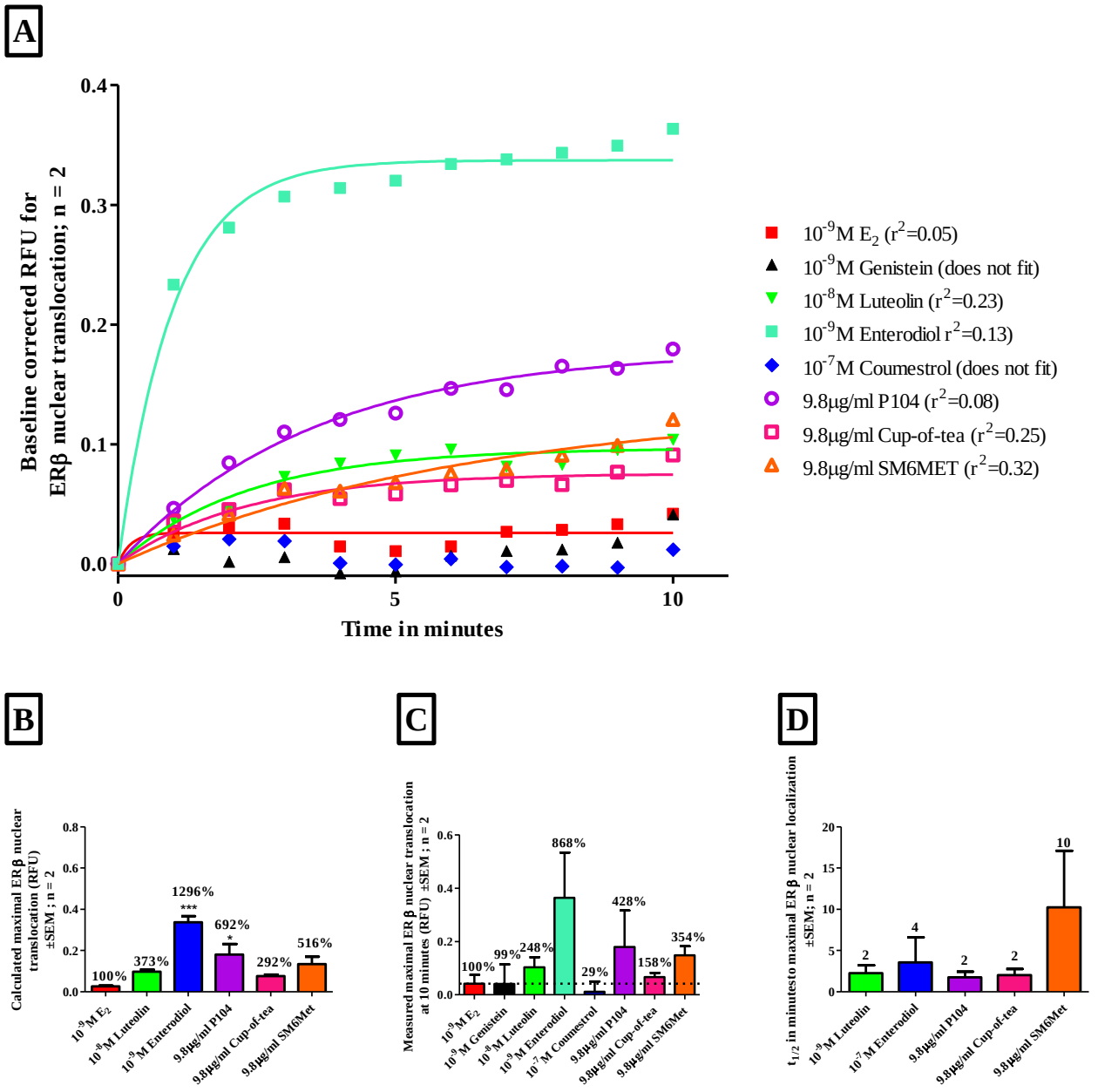


Figure 7. During a 10 minute period *Cyclopia* extracts, to a larger extent than E_2, concentrates ERβ in the nucleus, but the rate of ERβ import into the nucleus is slower than that of E_2. COS-1 cell were transiently transfected with YFP-ERβ and fluorescent intensity was monitored for 10 minutes with YFP images taken every 10 seconds. (A) Representative graph depicting changes in fluorescent intensity, within the ROI, over 10 minutes. Values for solvent control were baseline subtracted. Representative graphs depicting calculated maximal nuclear localization (B), measured maximal nuclear localization (C) and $t_{1/2}$ values for this period (D). The calculated maximal nuclear localization and $t_{1/2}$ values were determined with the GraphPad Prism® software fitting a one-phase association curve. Statistical analysis was done using One-way ANOVA with Dunnett's post-test comparing all columns to the E_2 treated cells (*, P<0.05; **, P<0.01; ***, P<0.001). Mean ± SEM is of two cells/treatment of two independent experiments. R^2 value depicts goodness of fit. (B-D) Dotted line through graphs represents 10^{-9}M E_2 values

Therefore, our combined data suggests that P104 and cup-of-tea result in a significantly faster ERα nuclear import rate than SM6Met, which displays a nuclear import rate similar to that of E_2. For ERβ (Fig. 7), calculated and measured maximal nuclear localization values obtained within the first 10 minutes of treatment were higher for all the *Cyclopia* extracts than for E_2. However, as similar results were only observed for P104 and SM6Met, but not cup-of-tea, at 30 minutes we can only definitively conclude that P104 and SM6Met treatment indeed resulted in greater ERβ maximal nuclear localization.

In conclusion, within the limits of our system, the methanol extracts of *Cyclopia* did not concentrate ERα in the nucleus to the same extent as E_2, however, ERβ was concentrated to a greater extent than for E_2. Furthermore, P104 and cup-of-tea, but not SM6Met, displayed a faster import rate for ERα maximal nuclear localization than E_2. Together these results suggest that in terms of nuclear localization, the methanol extracts of *Cyclopia* display ERα antagonist behaviour and ERβ agonist behaviour [13,18]. However, as our conclusions are limited to two biological repeats, it would be advantageous to obtain a third biological repeat as it may provide more statistical power to the observed changes after treatments.

4.3.5. Treatment with Cyclopia extracts reduced ordered nuclear distribution of YFP-ERα, while increasing ordered nuclear distribution of YFP-ERβ in COS-1 cells.

Upon ligand binding, nuclear receptors translocate to the nucleus and distributes in an orderly manner [18,50]. It has been shown, using green fluorescent protein tagged ER (GFP-ER), that upon agonist binding, the ER distributes in an ordered manner within the nucleus [18], while upon antagonist binding, a more random distribution of the ER is observed compared to agonist binding [18,19]. These studies, however, only evaluated the behaviour of the ERα subtype.

To study the nuclear distribution of ERα and ERβ, we used the method of Schaaf *et al.* [50] and Htun *et al.* [18]. In brief, this method entails transiently transfecting COS-1 cells with YFP-ERα/β, inducing with ligand, obtaining YFP images of the nucleus at desired time points, and drawing a long as possible straight line through the nucleus of a cell avoiding nucleoli (Fig. 8A). The coefficient of variation (CV) is then determined for the distribution of fluorescence intensity along this line (Fig. 8B). A high CV value signifies an ordered nuclear distribution, whereas a low CV value points to random nuclear distribution of ER. As this method is dependent on resolution, cells with a high as possible fluorescent resolution were chosen.

In Fig. 8A representative micrographs of a cell with an increased CV value over time (10^{-9}M E_2, ERα) and a cell with decreased CV over time (9.8 μg/ml cup-of-tea, ERα) are shown.

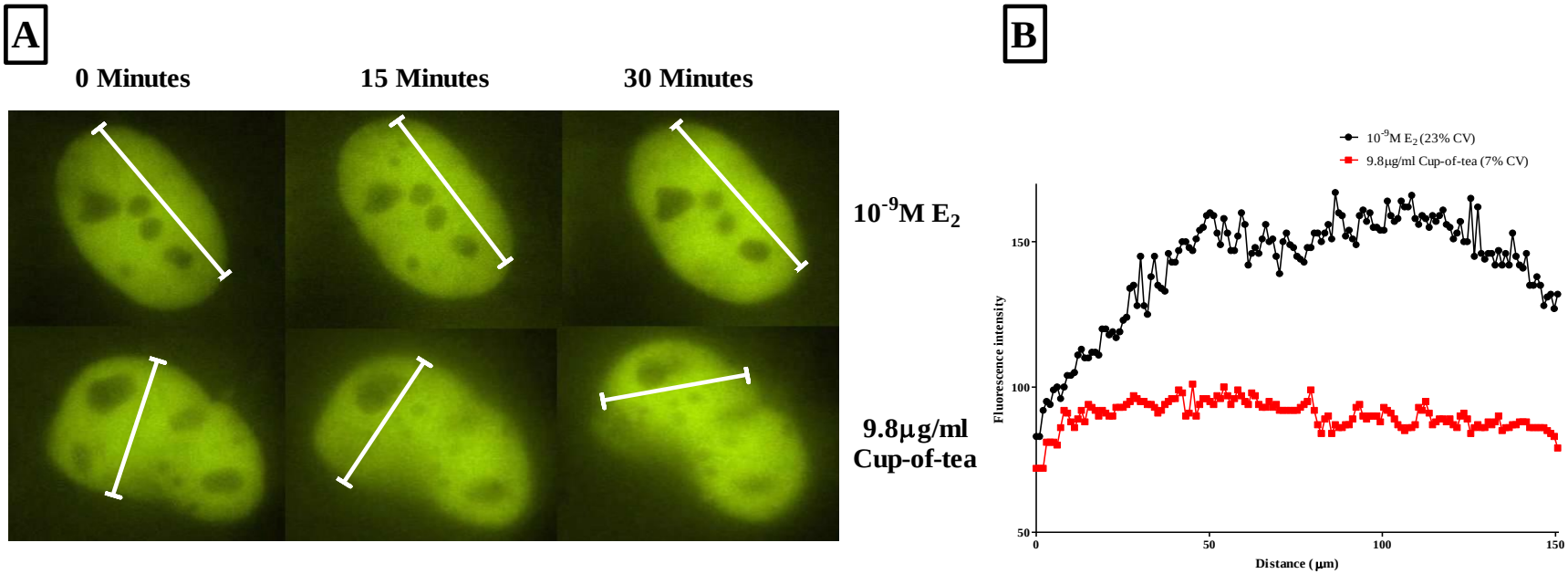


Figure 8. *Cyclopia* extracts reduced ordered nuclear distribution of YFP-ERα, whereas it increased ordered nuclear distribution of YFP-ERβ. COS-1 cells, transfected with either YFP-ERα or YFP-ERβ, were treated with E_2, polyphenols or *Cyclopia* extracts for 30 minutes. As long a line as possible (white line) was drawn within each nucleus, avoiding nucleoli and Cell® software was used to quantify fluorescent changes along this line and GraphPad Prism® software was used to calculate the CV of YFP fluorescent intensity. (A) Representative images of E_2 (high CV) and cup-of-tea (low CV) treated cells, transfected with YFP-ERα, at 0, 15, and 30 minutes. (B) Representative graph of fluorescent intensity changes along the white line for E_2 (23% CV) and cup-of-tea (7% CV) treated cells, transfected with YFP-ERα, at 30 minutes. (C&D) Coefficient of variation (CV) of cells transfected with either YFP-ERα (C) or YFP-ERβ (D) treated with either E_2, polyphenols, or *Cyclopia* extracts at 0, 15, and 30 minutes. Statistical analysis was done using One-way ANOVA with Dunnett's post-test comparing all columns to the 0 minute column of each treatment (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$) or the relevant E_2 column for each time point ($^{\#}$, $p<0.05$; $^{\#\#}$, $p<0.01$; $^{\#\#\#}$, $p<0.001$). Mean ± SEM is of two cells/treatment of two independent experiments.

The changes in fluorescent intensity along the white line drawn through cells (Fig. 9A) are shown in Fig. 8B (cells at 30 minutes). For ERα (Fig. 8C), E_2 and the polyphenol, genistein, significantly increased the CV value along the line after 30 minutes of treatment, thus depicting a more ordered nuclear distribution of ERα, suggesting that genistein behaved like an ERα agonist. In contrast, 30 minutes of treatment with the *Cyclopia* extracts induced a nuclear distribution significantly less ordered (significantly lower CV) than that of E_2, suggesting that the *Cyclopia* extracts act as ERα antagonists. No significant changes in CV values were obtained for ERβ (Fig. 8D). However, when comparing the 30 minute treatment values to the 30 minute value of the untreated control, a trend towards a higher CV value, and thus a more ordered nuclear distribution of ERβ, is observed for E_2, the polyphenols and the methanol extracts of *Cyclopia*, P104 and SM6Met. This ordered distribution is indicative of ERβ agonist activity [18,19]. In summary, the 30 minute treatment with *Cyclopia* extracts induced more random nuclear distribution of ERα than treatment with E_2, indicating ERα antagonist activity, whereas a trend to a more ordered nuclear distribution of ERβ in cells treated with the *Cyclopia* extracts is observed, like for E_2 treatment, indicating ERβ agonist activity.

4.4. Discussion

In their lifetime approximately 1 out of 87 women in the United States will develop invasive breast cancer [59]. The sex hormone, estrogen, which can affect cell viability, cell proliferation, and gene

expression [4,5], facilitates the development and growth of breast cancer [60,61]. Estrogens exert their function by binding to the ER [2,6,7], which exists as two subtypes, ERα and ERβ [8]. Furthermore, as ERβ has been shown to act as an inhibitor of ERα [20,23,26], which is associated with cell proliferation and breast cancer development [24], the modulation of the relative ER subtype protein levels, and thus also the biological processes under their control, has been identified as a molecular target in breast cancer and thus has become the focus of several strategies towards developing a treatment for the prevention and/or the treatment of breast cancer.

With regards to the ER and its subtype protein levels, SERDs have been developed [34,38-40]. Fulvestrant, a full ER antagonist, and GW5638/DPC974, a non-steroidal tamoxifen derivative [36,39], down-regulate ERα protein levels [34,35,39,40], while fulvestrant also stabilizes ERβ protein levels [34] and inhibits the growth of breast tumour xenografts [36,37]. In the current study we evaluated the effect of three phytoestrogenic extracts of *Cyclopia* on ER subtype protein levels, ERα:ERβ ratio, ER subtype nuclear localization and distribution.

The extracts of *Cyclopia*, like E_2, decreased the amount of ERα protein levels, while, unlike E_2, significantly increasing the ERβ protein levels over a 24 hour period in MCF-7BUS cells (Fig. 1). Concerning E_2 treatment, it is known that E_2 treatment down-regulates ERα protein levels [34,62-64] by enhancing ERα ubiquination and consequent ubiquitin-proteasome pathway mediated degradation in rats and MCF-7, PR1 Lactotrope, and transiently transfected HeLa cells [64-67]. Furthermore, consistent with our findings, E_2 slightly lowers ERβ protein levels in MCF-7 cells [34] and this decrease has also been shown to be mediated via the ubiquitin-proteasome pathway [68]. We may therefore postulate that the observed decrease in ERα protein levels by the methanol extracts of *Cyclopia*, P104 and SM6Met, is due to the ubiquination and consequent ubiquitin-proteasome pathway mediated degradation. However, this should be verified by repeating our experiment in the presence of proteasome inhibitors such as MG132, lactacystin or proteasome inhibitor I (PI) as GW7604, the

bioavailable metabolite of GW5638/DPC974, does not increase the ubiquination of ERα despite the fact that it down-regulates ERα [69].

To elucidate the mechanism whereby ERβ levels increased after treatment with *Cyclopia* extracts we look towards findings regarding fulvestrant, which stabilizes ERβ [34], and our results with polyphenols as we found that genistein, enterodiol, and coumestrol also significantly increased ERβ protein levels (Fig. 1). With regards to fulvestrant, not much is known about how it stabilizes ERβ protein levels, except that the residue D489 in helix 12 (responsible for co-activator interaction [70]) is important for conveying stability to the fulvestrant-ERβ complex [34]. Furthermore, binding of the SERM, tamoxifen, to ERα conveys stability to the tamoxifen-ERα complex due to hypoubiquitination of ERα [69]. Therefore, we may speculate that binding of fulvestrant and the *Cyclopia* extracts to ERβ may induce a conformational change that inhibits ubiquination of the receptor. This should be investigated. However, we should also evaluate whether, or not, the increase in ERβ protein levels after treatment with the *Cyclopia* extracts is a consequence of increased mRNA levels by using cycloheximide, an inhibitor of protein synthesis. With regards to our findings concerning the effect of the polyphenols on ERα and ERβ, it has been shown that in porcine granulosa cells originating from medium follicles, genistein has no significant effect on ERα protein and mRNA levels, while increasing ERβ protein and mRNA levels [71] validating our findings.

Although the most frequently occurring forms of breast cancer are both ERα and ERβ positive [27-29], during tumourigenesis the expression of ERβ is down-regulated, while ERα levels are up-regulated [72,73]. Therefore, the tempering effect ERβ on ERα [20,23,26,74] may be lost during breast cancer tumour progression when the ERα:ERβ ratio increases. Therefore, by combining qualitative Western blotting with quantitative whole cell binding (Figs. 2&3), we evaluated how treatment with E_2, polyphenols, or *Cyclopia* extracts changed the ERα:ERβ ratio (Table 1). We found that treatment with E_2 and the *Cyclopia* extracts decreased the ERα:ERβ ratio in comparison to solvent treatment.

However, this favourable ratio was achieved by different mechanisms with the *Cyclopia* extracts down-regulating ERα while also up-regulating ERβ, whereas E_2 only robustly down-regulated ERα (Fig. 1). Although the observed decrease in the ERα:ERβ ratio with the *Cyclopia* extracts were promising, as increased ERβ relative to ERα would result in increased inhibition of ERα-induced cell proliferation [75,76], the fact that E_2 resulted in a similar ratio, while associated with the promotion and growth of breast cancer [77], our result with E_2, depicting a more favourable ERα:ERβ ratio, is confusing. A possible scenario may involve effects of ER subtype levels on ER dimerization. It is known that the ER can dimerize to from ERα/α or ERβ/β homodimers or ERα/β heterodimers [27,58] with the ERα/α homodimer accelerating and the ERβ/β homodimer, as well as the ERα/β heterodimer, inhibiting breast cancer cell proliferation [57]. With regards to our findings, the down-regulation of ERα may lead to the abolishment of ERα/α homodimers. Furthermore, as ERα is the only heterodimeric partner capable of binding ligand to induce heterodimerization and relative similar amounts of both receptors are required for heterodimerization [27,57,58], we may postulate that the protective effect of the ERα/β heterodimer is abrogated with E_2 treatment as ERα protein levels are down-regulated by 77% after a 24 hour treatment period (Fig. 1). Furthermore, although the ERβ/β homodimer inhibits cell proliferation, it does elicit an increase in the transactivation of an ERE-containing promoter-reporter construct and, although this is lower than the induction observed with the ERα/α homodimer, this increase in transactivataion is higher than that observed with the ERα/β heterodimer [27,58]. Furthermore, ERβ can assume the function of ERα if ERα is absent [78] and ERβ can drive proliferation of mammary epithelial cells upon induction with the ERβ selective ligand BAG [79]. Thus, the possibility exists that the ERβ/β homodimer may drive processes that induce cell proliferation in the absence of ERα. Therefore we may speculate that the changes in ERα:ERβ ratio after E_2 treatment, abolishes heterodimerization, and that this loss drives E_2-induced proliferation of MCF-7BUS cells. In addition, we may also postulate that the ERα:ERβ ratio elicited by treatment with the *Cyclopia* extracts (all

higher ERα:ERβ ratios than E_2) may be more favourable for heterodimerization and that this may explain their lower potencies with regards to MCF-7BUS cell proliferation (Chapter 3). For future studies this hypothesis could be tested by using the bioluminescent resonance energy transfer (BRET) assay which allows for the detection of ERα/α and ERβ/β homodimers as well as ERα/β heterodimers [27]. An alternative hypothesis may involve the modulation of growth factors by the ER. It has been shown that ERα down-regulates growth factors, thus, ERα down-regulation by E_2 is accompanied by an increase of growth factors and their receptors [77,80] and this could explain the increase in cell proliferation when ERα is down-regulated. Furthermore, vascular endothelial growth factor (VEGF), a potent angiogenic factor in breast tumours, shown to be up-regulated by ERα and down-regulated by ERβ in MCF-7 cells [81], may be more significantly down-regulated in a scenario where both ERα is down-regulated and ERβ is up-regulated. Thus conferring an advantage to the *Cyclopia* extracts in comparison to E_2.

Although the ER is mostly nuclear, movement between the cytoplasm and the nucleus of the cell does take place [13-15]. Furthermore, having shown that *Cyclopia* extracts differently modulate the ER subtype levels, thereby changing the ERα:ERβ ratio, we wanted to investigate whether the extracts would alter the nuclear localization of the ER subtypes or disrupt movement between the cytoplasm and the nucleus and if there were differences in the behaviour of the subtypes. Using COS-1 cell transfected with either YFP-ERα or YFP-ERβ we found that the methanol extracts of *Cyclopia*, P104 and SM6Met, did not concentrate the ERα in the nucleus to the same extent as E_2 (Figs. 4&6). Furthermore the methanol extracts of *Cyclopia* also increased nuclear localization of ERβ to a greater extent than E_2 (Fig. 5&7). Furthermore, within the limitations of our test system, it does appear as if the *Cyclopia* extracts, specifically P104 and cup-of-tea, display a faster rate of ERα nuclear import than E_2. Therefore, in summary, our data suggests that the *Cyclopia* extracts are not as effective as E_2 at inducing nuclear localization of ERα into the nucleus despite the fact that the rate of nuclear

localization is faster, whereas the extracts are more efficient at inducing nuclear localization of ERβ into the nucleus. Previous findings regarding nuclear import and localization of the ER [13], also showed that although the majority of ER was already nuclear, an increase in nuclear ER was observed in transfected COS-1 cells upon E_2 treatment. However, upon treatment with the pure antiestrogens, fulvestrant and ICI 164,384, a decrease in nuclear ER was observed. Furthermore, Dauvois *et al.* [13] demonstrated that the pure antiestrogens disrupt shuttling of ER between the nucleus and cytoplasm. We may therefore speculate that the *Cyclopia* extracts, acting as ERα antagonists (Chapter 3), are not as effective as E_2 regarding the shuttling of ERα into the nucleus or, like the antiestrogens, may be partially disrupting the shuttling of ERα into the nucleus. In contrast, the *Cyclopia* extracts, acting as ERβ agonists (Chapter 3), may be increasing the movement of ERβ into the nucleus.

Decreased nuclear localization of ERα may be due to either reduced nuclear import or increased nuclear export of ERα. It has been proposed that heat shock protein (HSP) 70, associated with the ER [82], is involved in moving the ER across the nuclear membrane [13], therefore, it is possible that the *Cyclopia* extracts may be interfering with the HSP70-ERα interaction, thereby inhibiting nuclear uptake. Furthermore, it has been shown for the glucocorticoid receptor that treatment with a protein phosphatase inhibitor inhibits the retention of the glucocorticoid receptor in the nucleus [83]. Therefore, it may be possible binding of the *Cyclopia* extracts to ERα prevents the phosphorylation of ERα resulting in lower nuclear retention and increased export. Genistein, for example, has been shown to inhibit the nuclear export of the Bach1 protein by inhibiting dephosphorylation of Bach1 [84], while, in contrast, luteolin has been shown to inhibit the accumulation of HIF-1α in the nucleus by impairing its phosphorylation [85]. Therefore, it may be possible that *Cyclopia* extracts alter the nuclear localization of the ER by differentially affecting the phosphorylation status of the ER subtypes. However, further research, using inhibitors of phosphorylation as well as dephosphorylation is required.

Upon ligand binding and nuclear translocation the ER forms ordered clusters, indicative of areas of active transcription, within the cell nucleus [16-18]. While agonist binding results in an orderly distribution of nuclear receptors within the nucleus, antagonist binding, in contrast, induces a more random nuclear distribution [18,19,50]. We found that E_2, both an ERα and ERβ agonist, induced a more ordered distribution of both ERα and ERβ (indicated by higher CV value [50]). In contrast, the *Cyclopia* extracts, like fulvestrant, an ER antagonist [18], induced a more random nuclear distribution of ERα (lower CV value [50]), implying antagonism of ERα (Fig. 8). Furthermore, the *Cyclopia* extracts induced a slightly more ordered nuclear distribution of ERβ suggesting ERβ agonism.

To conclude, upon treatment with *Cyclopia* extracts, in MCF-7BUS cells, ERα protein levels were down-regulated while ERβ protein levels were up-regulated, resulting in an decreased ERα:ERβ ratio. Furthermore, treatment of COS-1 cells transfected with YFP-tagged ER with the *Cyclopia* extracts resulted in increased ERβ and decreased ERα nuclear localization. Furthermore, the *Cyclopia* extracts induced a more random nuclear distribution of ERα while inducing a more ordered distribution of ERβ. Together, these results support our previous findings that the extracts of *Cyclopia* act as ERα antagonists and ERβ agonists (Chapter 3) and suggest that the *Cyclopia* extracts may be behaving as subtype specific SERDs in down-regulating ERα while stabilizing ERβ protein levels.

Physiologically, considering the known roles of the ER subtypes in breast cancer development and progression [20-26,86,87], the down-regulation of ERα combined with the stabilization of ERβ may be considered a positive attribute of the *Cyclopia* extracts. In addition, the disruption of ERα nuclear localization with increased ERβ nuclear localization provides an additional mechanism whereby the proliferative action of ERα may be inhibited. Furthermore, the nuclear distribution of ERα and ERβ provides additional information regarding *Cyclopia* extracts as ERα antagonists and ERβ agonists, an attribute that may be beneficial for the development of an ideal drug for the treatment and/or prevention of breast cancer.

To conclude, our findings provide valuable insights into the mechanism whereby the phytoestrogenic extracts of *Cyclopia* modulate the proliferation of a human breast cancer cell line and, furthermore, provide additional proof that the extracts behave as ERα antagonists and ERβ agonists. Furthermore, these findings warrant further investigation that include, but is not limited to, *in vivo* breast cancer studies where we would not only monitor the development and progression of breast cancer tumours but also evaluate the levels and distribution of the ER subtypes present in the tumours and breast tissue.

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Chapter 5

Cyclopia extracts and estrogen elicit different responses relating to cancer promotion and progression in MCF-7BUS breast cancer cells.

5.1. Introduction

The development of breast cancer is not the result of one singular event, but rather the culmination of a series of events that may be divided into three steps namely, initiation, promotion, and progression [1-4]. During initiation, genomic DNA damage may occur and if this damage remains unrepaired, or is repaired incorrectly, it may lead to gene mutations that change the characteristics of a cell [4,5]. These gene mutations may convey survival and growth advantages to the cell and during promotion this cell can divide to form an actively proliferating cell population [4,6], which, during progression, gives rise to the production of tumour cells with increased proliferative capacity, invasiveness, and metastatic potential [4].

The proliferative capacity of a cell is determined by the cell cycle, which is the period between the formation of a new cell by the division of a mother cell and the point where this cell in turn divides to form two new daughter cells [7]. The mammalian cell cycle consists of several phases, the G0/G1 phase (gap phase), the S phase (synthesis phase), and the G2/M phase (gap and mitotic phase) [8-11]. The G0/G1 phase is the post-mitotic phase where the genomic integrity of the mother cell is checked and is a phase of cell growth where RNA and proteins are synthesized [9,12,13]. Furthermore, during the G0/G1 phase, DNA prereplication complexes are assembled that remain dormant until the S phase commences [14]. In addition, the G0/G1 phase is the only mitogenic dependent phase and after the cell is committed to enter the S phase, proliferation will continue independently of exogenous signals [15]. Furthermore, most of the cancer related defects that are initiated during the cell cycle occur during the G1 to S phase transition due to defective G1 phase control [16,17]. During the S phase, DNA is duplicated while protein and RNA synthesis continues [12,15]. The G2/M phase follows the S phase and during this phase DNA synthesis is halted, RNA and protein synthesis is reduced to a minimum, genomic stability is checked and the mother cell undergoes mitosis to generate two daughter cells [8,12,13]. Progression of cells through the cell cycle is coordinated at certain checkpoints and this

coordination allows for the necessary regulation of cell growth [18,19]. The loss of cell cycle checkpoint control is a hallmark of breast cancer development [15,20].

Estrogen (E_2), the natural female sex hormone [21-24], binds to the estrogen receptor (ER) and induces cell cycle progression via transcriptional up-regulation of cyclin D1 mRNA and protein levels [25]. Furthermore, activation of membrane associated ERα can transactivate epidermal growth factor receptor (EGFR) in breast cancer cells [26]. Activation of EGFR, in response to either steroid hormones or growth factors, activates Akt and mitogen-activated protein kinases (MAPKs), which up-regulate cyclin D1 thus promoting cell cycle progression [20,27]. Cyclin D1 binds and activates cell cycle dependent protein kinases and is important for cell cycle progression at the G1 to S phase checkpoint. Overexpression of cyclin D1 may lead to unrestricted cell proliferation and genomic instability [20,28], characteristics associated with cancer initiation and promotion. Thus, by up-regulating cyclin D1 levels, excessive estrogen signalling is associated with a cell acquiring these characteristics.

Furthermore, once the initiated cell has acquired increased proliferative capacity, it will continue to grow and divide to generate new highly proliferative clones with invasive and metastatic potential [4]. Tumour invasion and metastasis is a process whereby cancer cells from a primary tumour invade surrounding tissues and migrate to distant sites, thereby spreading cancer through the body [29-31]. In addition, metastasis at distant organs is the most common form of cancer reoccurrence and the foremost cause of fatalities in breast cancer patients [32,33], however, patients with ER positive tumours have a more favourable prognosis than patients with ER negative tumours [32], suggesting that the ER may play a protective role in breast cancer invasion and metastasis.

Previously we have shown that although phytoestrogenic extracts of *Cyclopia* (family: Fabaceae), an indigenous fynbos plant from the Western Cape province of South Africa [34,35], induced weak proliferation of the human breast cancer, MCF-7BUS cells, in the absence of E_2, they antagonised E_2-

induced proliferation of this cell line (Chapter 3). Therefore, considering the role of E_2 and its cognate receptor in the promotion and progression of breast cancer, as well as the effects of *Cyclopia* extracts on MCF-7BUS cell proliferation, we wanted to evaluate how the extracts of *Cyclopia* would affect the distribution of MCF-7BUS cells within the phases of the cell cycle. In addition, we also evaluated the effect of these extracts on the invasive capabilities of this cell line. Finally, as a key characteristic of cancer cells are growth independent of growth stimuli, due to either the modification, or overexpression, of growth factors or mutations of the components of the intracellular pathway transducing the stimulatory the signal [16,17,36,37], we also evaluated the effect of the *Cyclopia* extracts on the expression of genes involved in signal transduction, cell cycle, and apoptosis using the Human Breast Cancer RT^2 Profiler™ PCR Array. Furthermore, this array also allows for the evaluation of genes implicated in angiogenesis, adhesion, and proteolysis, which are all processes involved in cancer cell survival and invasion [30,31,38-41].

5.2. Materials and methods

5.2.1. Test Compounds

17β-Estradiol (E_2), genistein, luteolin, enterodiol, and fulvestrant (ICI 182,780) were obtained from Sigma-Aldrich®, South Africa, and coumestrol was obtained from Fluka™ Analytical, Sigma-Aldrich®, South Africa. The *Cyclopia* extracts, P104 [42], SM6Met [43] and cup-of-tea [43], were previously prepared. E_2, genistein, luteolin, enterodiol, coumestrol, ICI 182,780, and *Cyclopia* extract stock solutions were prepared in dimethylsulfoxide (DMSO). The concentrations of E_2 and the polyphenols used for experimental procedures in this chapter were chosen to reflect the concentrations that either displayed the highest efficacy in the absence of E_2 (polyphenols) or the strongest antagonistic effect on E_2 induction with the cell proliferation assay (Chapter 3).

5.2.2. Cell Culture

MCF-7BUS human breast cancer cells [44] (a kind gift from A. Soto, Tufts University, Boston, Massachusetts, United States of America) were maintained in high glucose (4.5 g/L) Dulbecco's modified eagle's medium (DMEM) (Sigma-Aldrich®) supplemented with 10% FCS (Highveld Biologicals, South Africa), 100 IU/ml penicillin and 100 µg/ml streptomycin (Gibco, Invitrogen™, South Africa), 2mM glutamine (Merck), 44mM sodium-bicarbonate (Gibco), 1mM sodiumpyruvate (Gibco), and 0.1mM non-essential amino acids (Gibco). All cells were maintained in a humidified cell incubator, set at 97% relative humidity and 5% CO_2 at 37˚C. Seven days prior to use, MCF-7BUS cells were withdrawn from 100 IU/ml penicillin and 100µg/ml streptomycin for seven days prior to use.

5.2.3. Cell cycle analysis

MCF-7BUS cells were seeded into sterile 10 cm tissue culture plates at a concentration of 1 x 10^6 cells/plate and allowed 24 hours to settle. After settling the cells were washed once with 10 ml pre-warmed PBS/plate and the medium was changed to DMEM without phenol red supplemented with 5% charcoal treated FCS (Highveld Biologicals) and incubated for 24 hours. Cells were then treated for 48 hours with E_2, polyphenols, and *Cyclopia* extracts where after nuclei were isolated and stained with propidium iodide (PI) with the CycleTEST™ PLUS DNA reagent kit (Becton Dickinson, South Africa) as described by the manufacturer. For excitation of PI stained nuclei a 488 nm solid state sapphire laser was used and emission was measured in the PE Texas Red channel on a linear scale using a 616/23 bandpass filter. PI stained nuclei emit fluorescent light at wavelengths between 580 and 650 nm. Fluorescent histograms were generated with the BD FACS Aria Cell sorter from Becton Dickinson, manufactured in San Jose, California, USA, using FACS Diva 6.1.3. software. To determine cell cycle phase distribution, fluorescence histograms were analysed using ModFit LTTM 3.0 software (Verity Software House, Topsham, Maine, USA).

5.2.4. Cell invasion assay

MCF-7BUS cells were seeded into sterile 10 cm tissue culture plates at a concentration of 1 x 10^6 cells/plate and allowed 24 hours to settle. After settling the cells were washed once with 10 ml pre-warmed PBS/per plate and the medium was changed to DMEM without phenol red supplemented with 5% charcoal treated FCS and incubated for 24 hours. The number of invasive cells was determined with the CytoSelect™ 96-Well cell invasion assay kit (Basement membrane, fluorometric format) (Cell Biolabs, Inc., BIOCOM biotech, South Africa) as described by the manufacturer. After steroid withdrawal the cells were reseeded into a 96 membrane chamber plate at a concentration of 5 x 10^5 cells/chamber in DMEM without phenol red containing either E_2, polyphenols, or *Cyclopia* extracts. The 96 membrane chamber plate was then placed in a feeder plate containing phenol red free DMEM supplemented with 10% charcoal treated FCS as chemoattractant. After a 24 hour incubation period at 37 °C, the 96 membrane chamber plate was removed from the tray containing the chemoattractant and placed in a tray containing the lysis buffer where cells, which had invaded the membrane, were dislodged from the bottom of the membrane, lysed and stained with CyQuant® GR dye (Invitrogen). Invasive cells were quantified by measuring fluorescence with a Thermo Scientific™ Varioskan plate reader at 480 nm/ 520 nm.

5.2.5. Microarray analysis

Microarray analysis was carried out using the Human Breast Cancer RT^2 Profiler™ PCR Array format E 384 (4 x 96) (Qiagen®, Whitehead Scientific (Pty) Ltd., South Africa, cat# PAHS-131Z). To prepare RNA samples MCF-7BUS cell were seeded into sterile 12 well tissue culture plates at a concentration of 1 x 10^5 cells/well. After settling the cells were washed once with 1 ml pre-warmed PBS/per plate and the medium was changed to DMEM without phenol red supplemented with 5% charcoal treated FCS and incubated for 24 hours. After incubation cells were treated with either E_2, genistein, or *Cyclopia* extracts for 6 hours. Total RNA was isolated from the treated MCF-7BUS cells using the

RNeasy Protect Cell Mini Kit (Qiagen®), treated with the RNase-Free DNase Set (Qiagen®) to eliminate genomic DNA contamination. cDNA was synthesised from 400 ng total RNA using the RT² First Strand Kit (Qiagen®). Following synthesis, cDNA was amplified and quantified using the RT² qPCR SYBR Green/ROX MasterMix-8 with an Applied Biosystems model 7900HT (384-well block) real-time cycler using the following cycling conditions: 10 minutes at 95 °C and 40 cycles of 15 seconds at 95 °C and 1 minute at 60 °C . Data was analysed using the Excel® SABiosciences PCR Array Data Analysis Template (www.SABiosciences.com/pcrarraydataanalysis.php). All kits were used according to the manufacturer's conditions.

5.2.6. Data manipulation and statistical analysis

The GraphPad Prism® version 5.10 for Windows (GraphPad Software) was used for graphical representations and statistical analysis. One-way ANOVA and Dunnett's post-test comparing all columns to either the solvent control or 10^{-9}M E_2 treatment were used for statistical analysis and significance is displayed on the graphs. For all experiments the error bars represent the SEM of at least three independent experimental repeats, except for the cell invasion assay where only one experiment was performed.

5.3. Results

5.3.1. In the absence of E_2 all Cyclopia extracts induce the accumulation of MCF-7BUS cells in the S phase of the cell cycle, whereas, in the presence of E_2, SM6Met induces the accumulation of MCF-7BUS cells in the G0/G1 phase of the cell cycle.

Previously (Chapter 3) we showed that although *Cyclopia* extracts induce weak proliferation of the MCF-7BUS breast cancer cell line, they inhibit E_2-induced cell proliferation. Thus we were interested in investigating the effects of the *Cyclopia* extracts on the cell cycle. Cell proliferation is dependent on the progression of cells through the cell cycle, which is coordinated at certain points, allowing for the necessary regulation of cell growth [18,19].

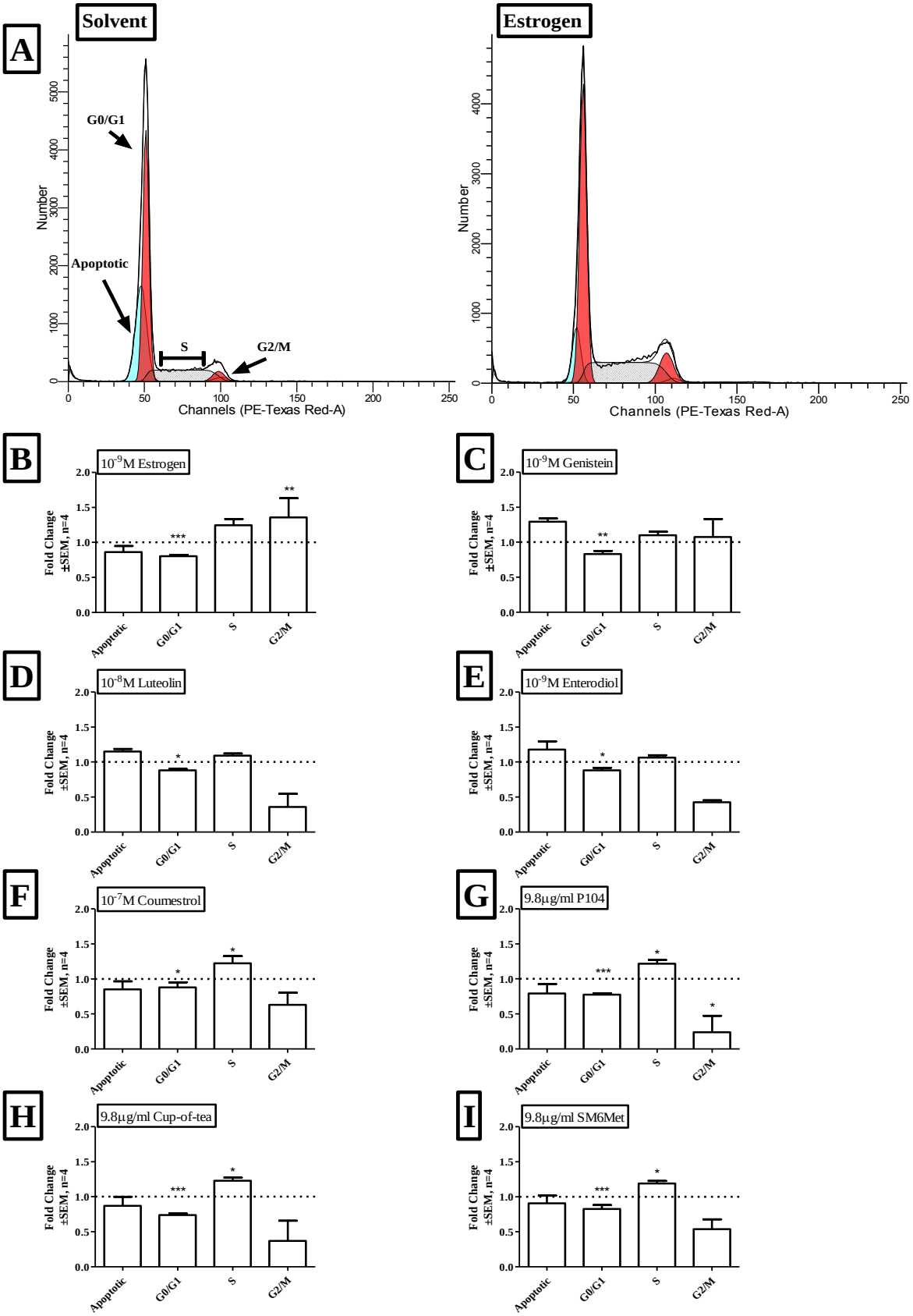


Figure 1. Extracts of *Cyclopia* induce the accumulation of MCF-7BUS cells in the S phase of the cell cycle in the absence of E_2. (A) Representative histograms of cell cycle distribution generated with FACS Diva 6.1.3. software for solvent and 10^{-9}M E_2 treated cells. (B-I) Representative curves of MCF-7 BUS cell cycle distribution following treatment with E_2 (B), polyphenols (C-F), or *Cyclopia* extracts (G-I) for 48 hours. Values were normalised to solvent control value within each individual experiment. Statistical analysis was done using One-way ANOVA with Dunnett's post-test comparing all values to the solvent control of the particular phase of the cell cycle (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$). The dotted line through the bars represents the values for solvent control. Mean ± SEM is of four independent experiments.

The mammalian cell cycle consists of several phases: The G0/G1 phase, which precedes the S phase and serves as a checkpoint for any DNA damage in the mother cell, and allows for cell growth; the S phase, also known as the synthesis phase, where DNA, which passed the G0/G1 checkpoint, is synthesised resulting in chromosome duplication; and the G2/M phase, which follows the S phase, is where duplicated DNA, from the S-phase, is checked for damage where after the cells enter mitosis [8,9,12,13,15]. Mitosis is followed by nuclear division, which is followed by cell division [7].

Using the CycleTEST™ PLUS DNA reagent kit, as described in the materials and methods section of this chapter, we evaluated the effect of the *Cyclopia* extracts on the distribution of cells between the different phases of the cell cycle, both in the absence (Fig. 1) and presence (Fig. 2) of 10^{-9}M E_2. Representative histograms of solvent and E_2 treated cells are shown (Fig. 1A) and phases of the cell cycle, as represented by the histogram, are indicated with arrows. Using ModFit LTTM 3.0 software the percentage of apoptotic cells were also determined (Fig. 1A). Treatment with E_2 (Fig. 1B) significantly decreased the number of cells present in the G0/G1 phase and significantly increased the number of cells in the G2/M phase when compared to solvent treated cells (dashed line). An increase in the number of cells present in the S phase as well as a small decrease in the number of apoptotic cells is seen, although these changes are not significantly different from the solvent values. Treatment with the *Cyclopia* extracts (Figs. 1G, H, & I) extracts, like E_2, significantly decreased the number cells in the G0/G1 phase and increased the number of cells in the S phase of the cell cycle. However, unlike E_2, the *Cyclopia* extracts decreased the number of cells in the G2/M phase of the cell cycle.

Table 1. The effect of E_2, polyphenols, and *Cyclopia* extracts on the distribution of human breast cancer cells within the cell cycle. MCF-7 BUS cells were treated with E_2, polyphenols, or *Cyclopia* extracts for 48 hours. After treatment the percentage of cells in each phase of the cell cycle was determined. Statistical analysis was done using One-way ANOVA with Dunnett's post-test comparing all values to the solvent control of the particular phase of the cell cycle. Mean ± SEM is of four independent experiments.

	Percentage of cells in cell cycle phase							
	Apoptotic	**Change[a]**	**G0/G1**	**Change**	**S**	**Change**	**G2/M**	**Change**
Solvent	28.59 ± 1.52		48.50 ± 7.79		44.74 ± 9.15		2.67 ± 0.47	
10^{-9}M E_2	20.36 ± 2.22	-8.23[b]	39.97 ± 4.28	-8.53	51.19 ± 5.97	6.45	6.94 ± 1.72	4.27
10^{-9}M Genistein	36.14 ± 3.34	7.55	47.86 ± 9.92	-0.64	51.17 ± 17.41	6.43	2.33 ± 0.40	-0.34
10^{-8}M Luteolin	28.32 ± 2.34	-0.27	41.01 ± 11.09	-7.49	50.17 ± 10.88	5.43	1.29 ± 0.59	-1.38
10^{-9}M Enterodiol	29.34 ± 4.46	0.75	42.48 ± 13.45	-6.02	49.64 ± 11.73	4.90	1.56 ± 0.51	-1.11
10^{-7}M Coumestrol	21.80 ± 2.68	-6.79	48.00 ± 7.95	-0.50	47.00 ± 10.23	2.26	1.75 ± 0.74	-0.92
9.8µg/ml P104	25.32 ± 4.04	-3.27	36.02 ± 9.78	-12.48	55.06 ± 10.88	10.32	1.27 ± 1.26	-1.40
9.8µg/ml Cup-of-tea	22.54 ± 3.22	-6.05	34.58 ± 9.85	-13.92	55.72 ± 11.22	10.98	1.83 ± 1.62	-0.84
9.8µg/ml SM6Met	24.65 ± 3.25	-3.94	36.52 ±10.63	-11.98	54.22 ± 11.19	9.48	2.44 ± 1.20	-0.23

[a]Change from solvent value.
[b]Negative value denotes a decrease in percentage cells.

The polyphenols, luteolin, enterodiol, and coumestrol (Figs. 1D-F), behaved like the *Cyclopia* extracts, while genistein (Fig. 1C) displayed a similar cell cycle distribution profile as E_2. Genistein, luteolin, and enterodiol, unlike E_2 and the *Cyclopia* extracts, increased the number of apoptotic cells, although the values did not reach significance. Whereas Figure 1 displayed the average normalized values relative to solvent control within each individual experiment Table 1 shows average values of the percentage MCF-7BUS cells in each phase of the cell cycle for all treatments in the absence of 10^{-9}M E_2. Table 1 shows similar trends to that obtained with the normalized values, although the values did not reach significance.

In the presence of 10^{-9}M E_2 (Fig. 2), no significant deviations from the E_2-induced effect (dashed line) was observed, except for SM6Met (Fig. 2G), which significantly increased the number of cells in the G0/G1 phase. All other treatments (Figs. 2A-F) increased the number of apoptotic cells, although the values did not reach significance. Furthermore, upon comparison of the distribution patterns of MCF-7BUS cells within the cell cycle following treatment with the *Cyclopia* extracts in the presence of E_2 to the distribution patterns of solvent treated cells (Fig. 3, dashed line equals solvent), we observed a trend for SM6Met towards redistribution of the cells to the basal distribution (Fig 3H). However, the P104 and cup-of-tea extracts did not appear to alter the E_2-induced MCF-7BUS cell distribution significantly (Figs. 3F&G). The polyphenols, genistein (Fig. 2A) and enterodiol (Fig. 2C), increased the number of cells in the G2/M phase of the cell cycle, whereas luteolin (Fig. 2B), coumestrol (Fig. 2D), and the *Cyclopia* extracts, P104 (Fig. 2E) and SM6Met (Fig. 2F), decreased the number of cells in the G2/M phase when compared to E_2, although none of the values reached significance. In addition, none of the polyphenols altered the E_2-induced MCF-7BUS cell distribution (Figs. 3B,D&E) although, luteolin did decrease the number of cells in the G2/M phase, however this value did not reach significance (Fig. 3C).

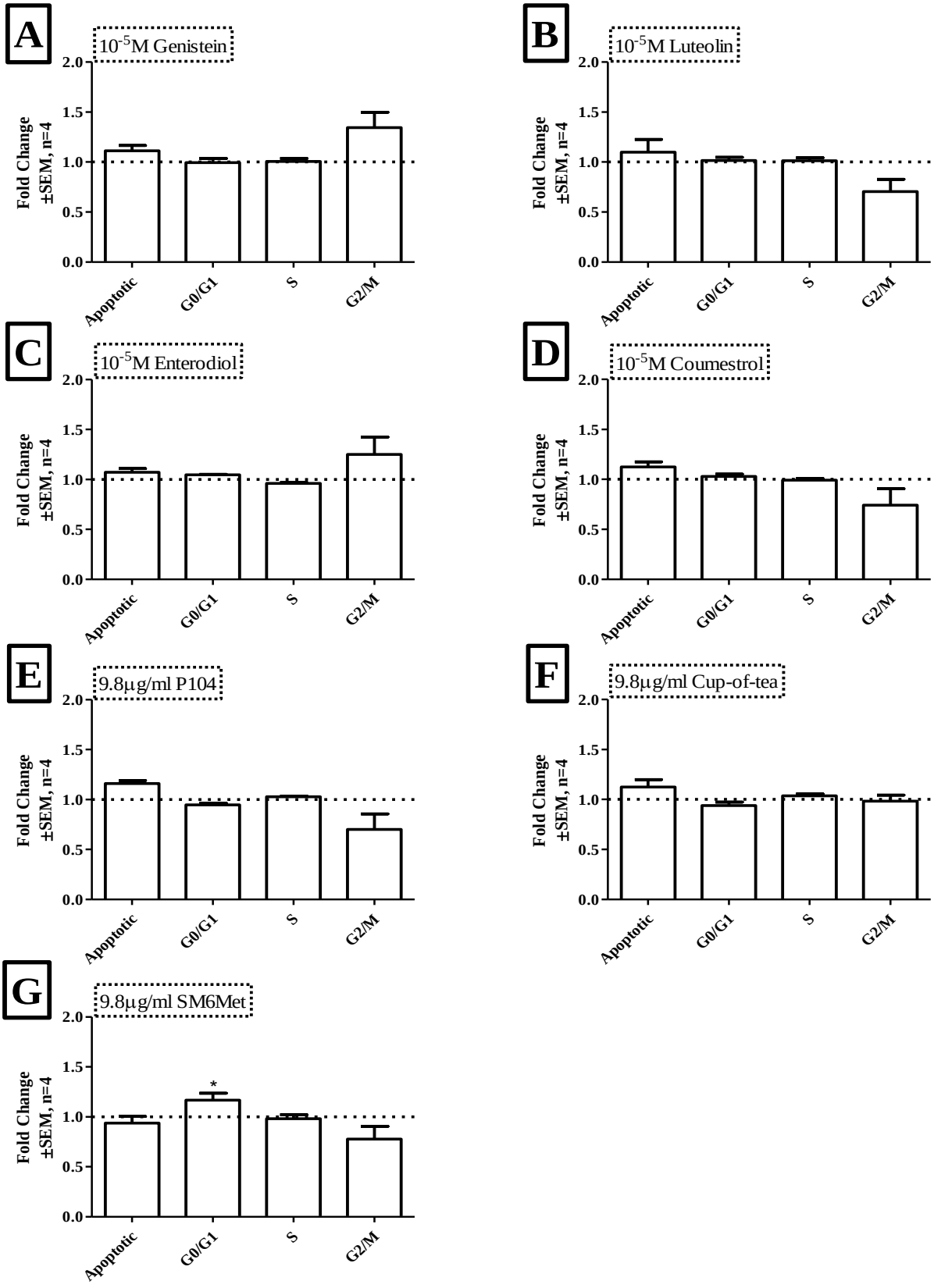


Figure 2. The *Cyclopia* extract, SM6Met, induces the accumulation of MCF-7BUS cells in the G0/G1 phase of the cell cycle in the presence of E_2. Representative curves of MCF-7 BUS cell cycle distribution following treatment with polyphenols (A-D) or *Cyclopia* extracts (E-G) for 48 hours in the presence of 10^{-9}M E_2. Values were normalised to 10^{-9}M E_2 value within each individual experiment. Statistical analysis was done using One-way ANOVA with Dunnett's post-test comparing all values to 10^{-9}M E_2 treated value of the particular phase of the cell cycle (*, P<0.05; **, P<0.01; ***, P<0.001). The dotted line through the bars represents the values for 10^{-9}M E_2. Mean ± SEM is of four independent experiments.

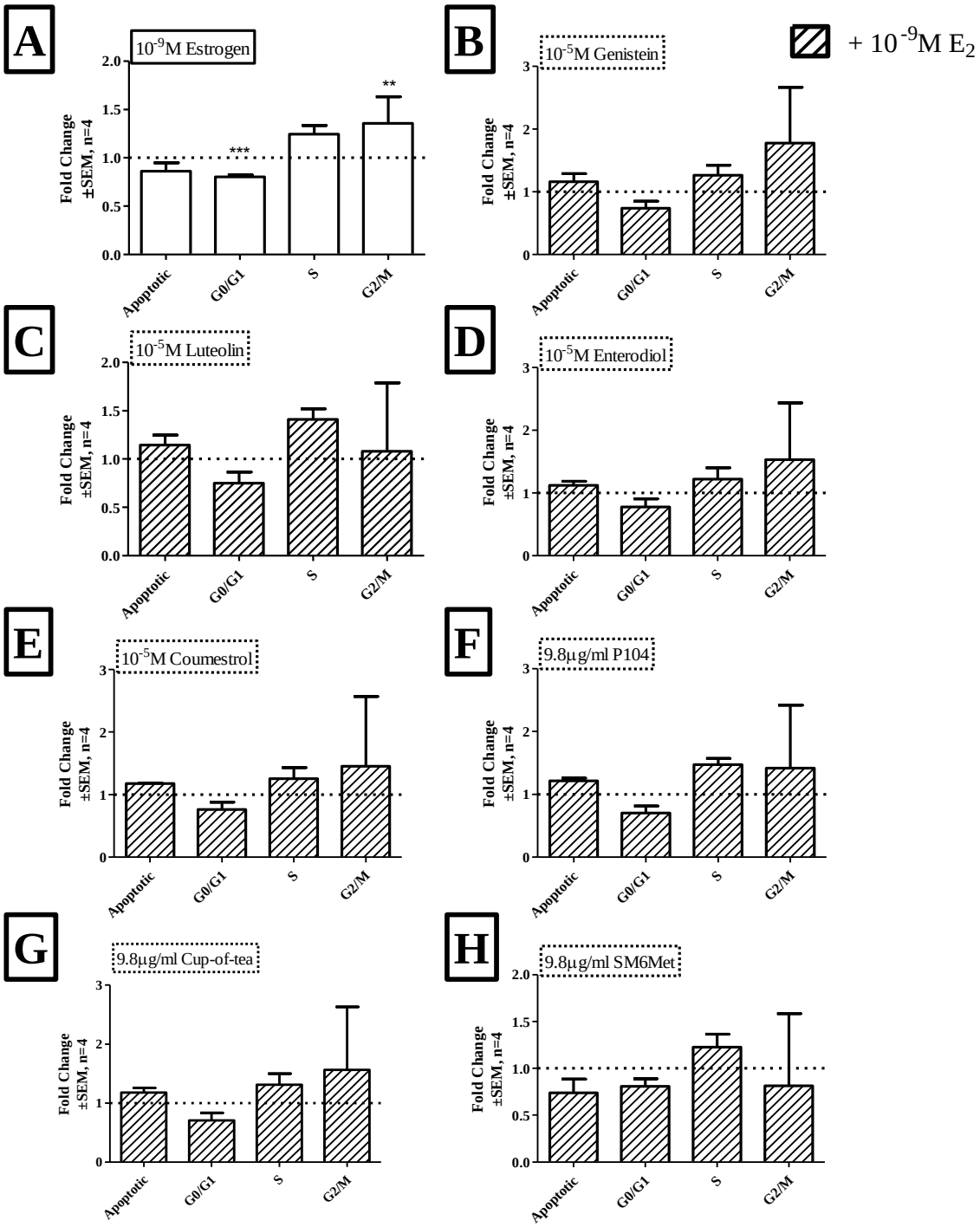


Figure 3. In the presence of E_2, the *Cyclopia* extract, SM6Met, redistributes E_2-induced MCF-7BUS cell cycle phase distribution towards basal levels. Representative curves of MCF-7 BUS cell cycle distribution following treatment with E_2 (A) or polyphenols (B-E) or *Cyclopia* extracts (F-H) for 48 hours in the presence of 10^{-9}M E_2. Values were normalized to solvent control values. Statistical analysis was done using One-way ANOVA with Dunnett's post-test comparing all values to solvent treated value of the particular phase of the cell cycle (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$). The dotted line through the bars represents the values for solvent. Mean ± SEM is of four independent experiments.

Whereas Figures 2 and 3 displayed the average normalized values relative to 10^{-9}M E_2 and solvent control values, respectively, within each individual experiment, Table 2 shows average values of the percentage MCF-7BUS cells in each phase of the cell cycle for all treatments in the presence of 10^{-9}M E_2. Table 2 shows similar trends to that obtained with the normalized values, although the values did not reach significance.

In conclusion, in the absence of 10^{-9}M E_2 (Fig. 1), the extracts of *Cyclopia*, like E_2, induced redistribution of cells from the G0/G1 to the S phase of the cell cycle, but had an opposite effect to that of E_2 in the distribution of cells in the G2/M phase, with the extracts decreasing rather than increasing the number of cells in the G2/M phase. In the presence of E_2, the SM6Met extract of *Cyclopia* significantly increased the number of cells in the G0/G1 phase of the cell cycle relative to E_2 (Fig. 2) and trended towards redistributing the MCF-7BUS cells towards a basal level distribution (Fig. 3).

Table 2. The effect of polyphenols and *Cyclopia* extracts on the distribution of human breast cancer cells within the cell cycle in the presence of E_2. MCF-7 BUS cells were treated with polyphenols or *Cyclopia* extracts for 48 hours in the presence of 10^{-9}M E_2. After treatment the percentage of cells in each phase of the cell cycle was determined. Statistical analysis was done using One-way ANOVA with Dunnett's post-test comparing all values to the 10^{-9}M E_2 values of the particular phase of the cell cycle (*, P<0.05; **, P<0.01; ***, P<0.001). Mean ± SEM is of four independent experiments.

	Percentage of cells in cell cycle phase							
	Apoptotic	**Change[a]**	**G0/G1**	**Change**	**S**	**Change**	**G2/M**	**Change**
10^{-9}M E_2	20.36 ± 2.22		39.97 ± 4.28		51.19 ± 5.97		6.94 ± 1.72	
+ 10^{-5}M Genistein	22.69 ± 4.12	2.33	39.85 ± 4.97	-0.12[b]	55.33 ± 6.30	4.14	10.24 ± 2.51	3.30
+ 10^{-5}M Luteolin	28.16 ± 2.56	7.80	38.33 ± 5.71	-1.64	58.30 ± 7.34	7.11	6.51 ± 3.11	-0.43
+ 10^{-5}M Enterodiol	27.66 ± 1.56	7.30	39.74 ± 6.57	-0.23	56.11 ± 8.26	4.92	6.81 ± 3.41	-0.13
+ 10^{-5}M Coumestrol	28.92 ± 0.27	8.56	38.92 ± 5.89	-1.05	57.67 ± 8.10	6.48	6.64 ± 4.80	-0.30
+ 9.8µg/ml P104	29.84 ± 1.09	9.48	35.89 ± 5.58	-4.08	60.79 ± 7.56	9.60	6.53 ± 4.01	-0.41
+ 9.8µg/ml Cup-of-tea	28.96 ± 1.95	8.60	36.04 ± 6.59	-3.93	60.30 ± 8.56	9.11	7.33 ± 3.95	0.39
+ 9.8µg/ml SM6Met	18.12 ± 3.66	-2.24	43.76 ± 4.61	3.79	56.29 ± 6.40	5.10	5.08 ± 4.71	-1.86

[a]Change from solvent value.
[b]Negative value denotes a decrease in percentage cells.

5.3.2. The methanol extracts of Cyclopia, P104 and SM6Met, like the ER antagonist ICI 182,780, increased the number of invasive MCF-7BUS cells in the presence of E_2.

Tumour invasion, a hallmark of cancer associated with progression [30], is a process whereby cancer cells from a primary tumour invade surrounding tissues and migrate to distant sites thereby spreading cancer through the body [29]. Therefore, we wanted to evaluate the effect the *Cyclopia* extracts on the number of invasive MCF-7BUS BC cells, both in the presence and absence of 10^{-9}M E_2.

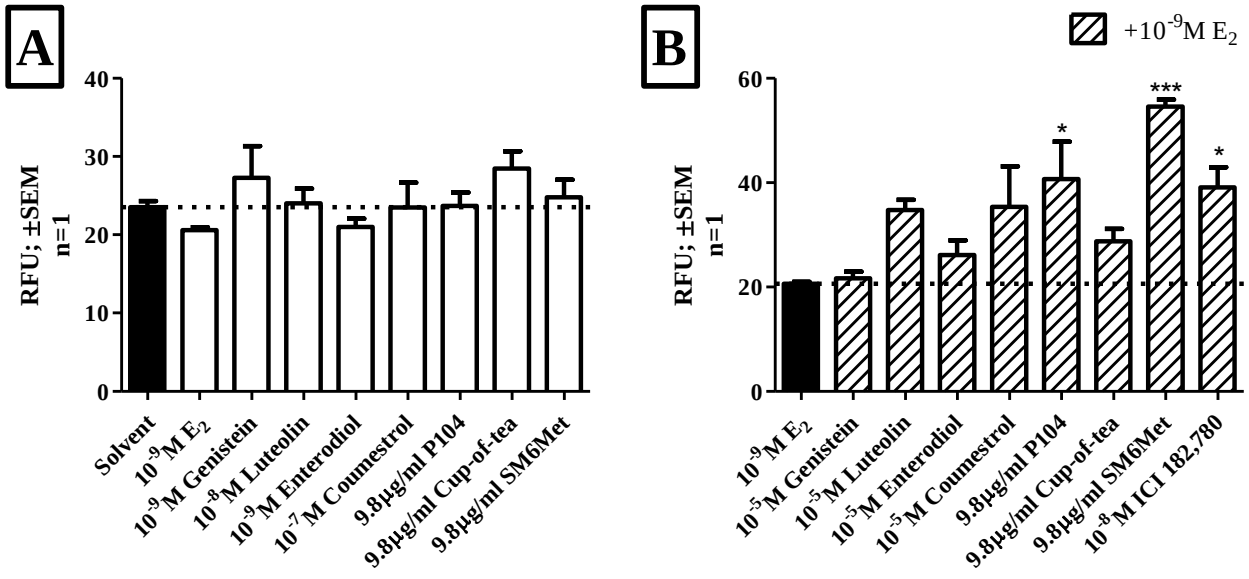


Figure 4. The number of invasive MCF-7BUS cells is increased by the *Cyclopia* extracts, P104 and SM6Met, in the presence of 10^{-9}M E_2. The number of invasive MCF-7BUS cells was determined using the CytoSelect™ 96-Well cell invasion assay kit as described in the material and methods section. Figures represent (A) the effect of E_2, polyphenols, or *Cyclopia* extracts in the absence of E_2 and (B) in the presence of E_2 on the number of invasive cells. Statistical analysis was done using One-way ANOVA with Dunnett's post-test comparing all values to the (A) solvent control or (B) 10^{-9}M E_2 treated cells. Mean ± SEM is of one experiment with five replicates.

No significant changes in the number of invasive cells were observed after treatment with E_2, the polyphenols, or the *Cyclopia* extracts alone (Fig. 4A). A slight decrease in the number of invasive cells was observed after E_2 treatment, whereas the polyphenol, genistein, and the *Cyclopia* extracts, cup-of-tea and SM6Met, slightly, but not significantly, increased the number of invasive cells.

In the presence of 10^{-9}M E_2 (Fig. 4B), the *Cyclopia* extracts, P104 and SM6Met, like the full ER antagonist, fulvestrant (ICI 182,780), significantly increased the number of invasive cells when compared to cells treated with E_2. The polyphenols, luteolin, enterodiol, and coumestrol, also increased the number of invasive cells, however, not significantly when compared to E_2 treated cells.

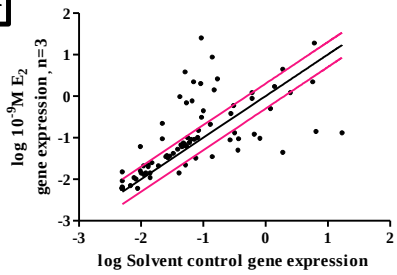
To summarize, the *Cyclopia* extracts had no significant effect on the number of invasive MCF-7BUS cells in the absence of E_2, but, like fulvestrant, P104 and SM6Met increased the number of invasive cells in the presence of 10^{-9}M E_2.

5.3.3. PCR array analysis of MCF-7BUS cells revealed that treatment with the Cyclopia extracts generate gene expression patterns that differ from that of E_2 and furthermore, within the group of Cyclopia extracts, extracts from different species regulate genes in a different way.

Hanahan *et al.* [30] refers to cancer cells as "masters of their own destinies" as they acquire the ability to grow without stimulation by modifying growth factors, their expression levels, or components of the intracellular signal transduction pathway [16,17,36,37]. Therefore, having evaluated the effect of the *Cyclopia* extracts on MCF-7BUS cell cycle distribution well as the effect on MCF-7BUS cell invasion, we evaluated the effect of the *Cyclopia* extracts on the expression of genes associated with signal transduction, the cell cycle, and apoptosis, as well as genes that may influence cancer cell survival and invasion via angiogenesis, epithelial to mesenchymal transition (EMT), adhesion and proteolysis, using the Human Breast Cancer RT^2 Profiler™ PCR Array.

Using this PCR array, which focuses on genes involved in signal transduction, cell cycle progression, apoptosis, angiogenesis, adhesion, and proteolysis in breast cancer, as described in the materials and methods section, we investigated how E_2, genistein, and *Cyclopia* extracts modulated the transcription of genes associated with breast cancer (specific gene regulation results are shown in Table S1).

A



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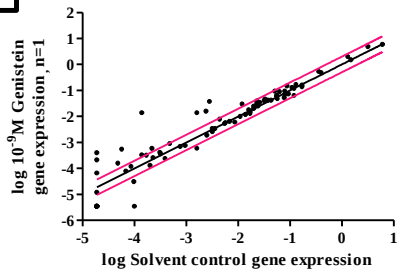

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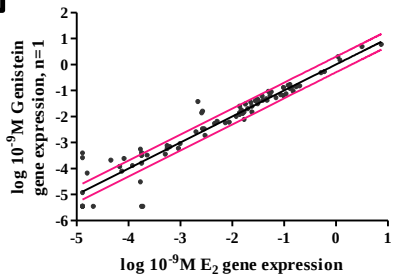

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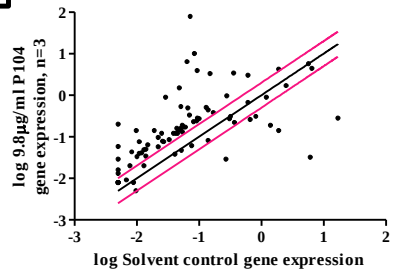

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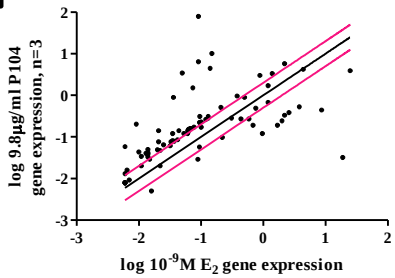

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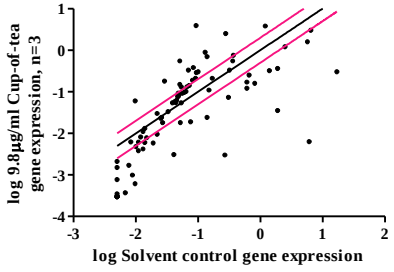

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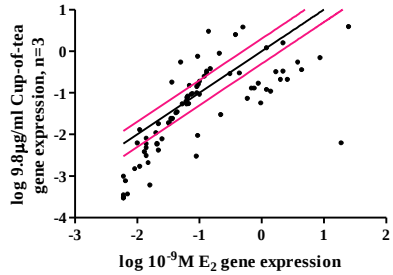

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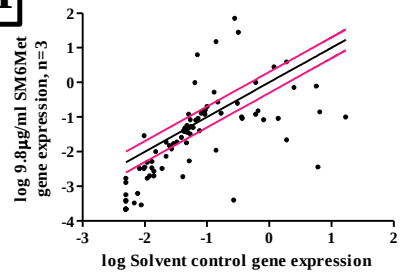

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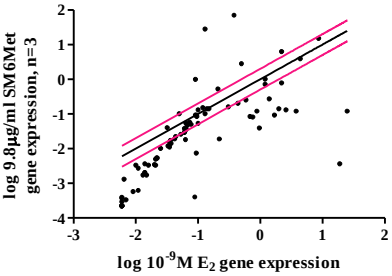

Figure 5. Treatment of MCF-7BUS cells with *Cyclopia* extracts generate gene expression patterns that differ from that of E_2. Changes in gene expression patterns in MCF-7BUS cells after treatment with 10^{-9}M E_2 (A), 10^{-9}M genistein (B&C), 9.8µg/ml P104 (D&E), 9.8µg/ml cup-of-tea (F&G), and 9.8µg/ml SM6Met (H&I) relative to the gene expression pattern in both the solvent (A, B, D, F, & H) and E_2 treated cells(C, E, G, & I). The pink lines represent ± two times fold change in gene expression levels and the black line represents no change from either solvent (A, B, D, F, & H) or E_2 treated (C, E, G, & I) cells. Figures are of log mean fold change in gene expression relative to housekeeping genes (ACTB, B2M, GAPDH, HPRT1, RPL13A) and are representative of three independent experiments, except for genistein, which represents only one experiment.

An overview of the global gene expression pattern, compared to solvent (Figs. 5A, B, D, F, & H) or 10^{-9}M E_2 (Figs. 5C, E, G, & I), may be achieved by generating log-log plots of average fold change in gene expression relative to the housekeeping genes, where the black line indicates no change in gene expression and the pink lines indicate ± two fold change in gene expression (generally accepted cut-off for significant changes in fold gene expression [45-47]). Genes positioned above the pink line in the top left quadrant were up-regulated more than two fold relative to the treatment on the x-axis, while those below the pink line in the bottom right quadrant were down-regulated more than two fold. Treatment with 10^{-9}M E_2 (Fig. 5A) generated a global expression pattern where a similar number of genes were up-regulated and down-regulated relative to solvent (23.81% up-regulated vs. 19.05% down-regulated (Table 3)). Upon treatment with the *Cyclopia* extract, P104 (Fig. 5D), the global gene expression pattern indicated that the majority of genes regulated by this extract were up-regulated relative to the solvent control (65.48% up-regulated vs. 10.71% down-regulated). Treatment with the cup-of-tea (Fig. 5F) and SM6Met (Fig. 5H) *Cyclopia* extracts, however, unlike with E_2 and P104, resulted in the majority of the genes being down-regulated (21.43% up-regulated vs. 45.24% down-regulated and 16.67% up-regulated vs. 46.43% down-regulated, respectively) relative to solvent control (Table 3). Treatment with the polyphenol, genistein (Fig. 5B), unlike with E_2 and the *Cyclopia* extracts, resulted in the majority of genes not being regulated more than ± two fold relative to solvent (72.61%) and, of the regulated genes, similar numbers were up- and down-regulated (14.29% up-regulated vs. 13.10% down-regulated) (Table 3), however caution should be exercised regarding genistein results as they represent only one experiment.

Therefore, compared to the global gene expression pattern of the solvent control treated cells, the *Cyclopia* extracts affected more genes than either E_2 or genistein (63-76% vs. 43% and 27%, respectively) (Table 3), which may reflect the fact that the extracts, in contrast to E_2 and genistein, represent the contribution of several potential phytoestrogenic compounds (Chapter 3 and Addendum A [48]). Furthermore, treatment with P104, a *C. genistoides* extract, generated a global gene expression pattern quite similar to that of E_2 (Figs. 5A&D), whereas the extracts of *C. subternata*, cup-of-tea and SM6Met, generated global gene expression patterns that, although similar to each other (Figs. 5F&H), differ from that of E_2 and P104. Also, upon treatment with P104, the majority of the genes were up-regulated, whereas with cup-of-tea and SM6Met, the majority were down-regulated (Table 3).

In addition, we plotted the global gene expression values of genistein and the *Cyclopia* extracts against that of E_2 (Figs. 5C, E, G, & I). The global gene expression pattern for P104 became more condensed with the majority of genes falling on, or just above, the plus two fold change in fold gene expression line (Fig. 5E), supporting our previous suggestion that, relevant to solvent, P104 and E_2 generate quite similar gene expression patterns, although more genes were up-regulated by P104 than by E_2. Furthermore, for the cup-of-tea (Fig. 5G) and SM6Met (Fig. 5I) extracts, relative to E_2, as for relative to solvent, a larger number of genes were found in the lower right hand quadrant of the graph, suggesting that, not only did treatment with the cup-of-tea and SM6Met extracts lower the expression levels of genes when compared to that of solvent, but also when compared to that of E_2. Furthermore, when the expression patterns of genistein regulated genes were compared to that of E_2 regulated genes (Fig. 5C), the majority of genes were not regulated by more than ± two fold relative to E_2, however, as mentioned previously, caution should be exercised regarding genistein results as they represent only one experiment

Table 3. Extracts of Cyclopia regulate both common and distinct genes within functional groups. MCF-7BUS cells were treated with E_2, genistein and *Cyclopia* extracts. Genes were grouped according to their function in the development and progression of breast cancer. Regulation was determined as ≥ or ≤ than 2 fold change from solvent control. Values are representative of three independent experiments, except for genistein, which represents only one experiment.

	10^{-9} M E_2		10^{-9}M Genistein		9.8 µg/ml P104		9.8 µg/ml Cup-of-tea		9.8 µg/ml SM6Met	
	↑[a]	↓[b]	↑	↓	↑	↓	↑	↓	↑	↓
Overall gene regulation [84][c]	23.81[d]	19.05	14.29	13.10	65.48	10.71	21.43	45.24	16.67	46.43
Functional grouping										
Angiogenesis [14]	7.14	35.71	21.43	0.00	64.29	21.43	14.29	50.00	14.29	64.29
Epithelial to mesenchymal transition [5]	20.00	20.00	20.00	0.00	80.00	20.00	20.00	20.00	20.00	20.00
Adhesion [13]	23.08	23.08	15.38	0.00	61.54	23.08	7.69	38.46	23.08	61.54
Proteolysis [7]	14.29	0.00	28.57	14.29	71.43	14.29	14.29	71.43	14.29	85.71
Apoptosis [20]	15.00	20.00	25.00	15.00	60.00	10.00	10.00	45.00	15.00	60.00
Cell cycle [18]	27.78	27.78	5.56	5.56	66.67	16.67	22.22	44.44	16.67	55.56
DNA damage [12]	25.00	8.33	8.33	0.00	91.67	8.33	8.33	8.33	16.67	25.00
Xenobiotic transport [2]	0.00	0.00	0.00	50.00	50.00	0.00	0.00	50.00	0.00	50.00
Transcription factors [17]	29.41	23.53	17.65	5.88	64.71	23.53	23.53	47.06	11.76	41.18

[a]Percentage of genes up-regulated relative to total number of genes within group.
[b]Percentage of genes down-regulated relative to total number of genes within group.
[c]Total number of genes in group.
[d]Percentage calculated: [number of genes regulated (≥ ± 2 fold change)] / (total number of genes in group)*100

Therefore, to summarise, the extracts of *Cyclopia* regulated the global gene expression levels of genes associated with signal transduction, cell cycle progression, apoptosis, angiogenesis, adhesion, and proteolysis in breast cancer. Furthermore, the global gene regulation patterns were different for extracts of different *Cyclopia* species, with the extracts of *C.genistoides* increasing the expression levels of the majority of genes, whereas the extracts of *C. subternata* decreased the expression levels of the majority of the regulated genes.

To evaluate the number of genes that are similarly or differentially regulated by different treatments we generated Venn diagrams depicting commonly or distinctly regulated genes between the *Cyclopia* extracts (Fig. 6A), P104, E_2, and genistein (Fig. 6B), cup-of-tea, E_2, and genistein (Fig. 6C), and SM6Met, E_2, and genistein (Fig. 6D). Upon comparison of the three *Cyclopia* extracts (Fig. 6A) 27 of the 84 genes were commonly regulated with 59% of these genes regulated in the same direction (all treatments either up- or down-regulated gene expression) by all three extracts, while 41% were regulated in different directions (the same gene up-regulated by one extract but down-regulated by the other two extracts, or *vice versa*). P104 was the outlier responsible for 82% of the differentially regulated genes, again illustrating afore mentioned differences in behaviour of the extracts from the different species of *Cyclopia*. Out of the 84 genes P104, cup-of-tea, and SM6Met distinctly regulated 16, 4, and 1 gene, respectively, within this comparison. Of the 84 genes, 10 genes were regulated by both P104 and cup-of-tea, but not SM6Met, 90% of which were regulated in the same direction and 10% in different directions. Furthermore, 11 genes were regulated by P104 and SM6Met, but not cup-of-tea, and 36% were regulated in the same and 64% in different directions. The cup-of-tea and SM6Met extracts both regulated 15 genes that were not regulated by P104, 93% were regulated in the same direction and only 7% in different directions, again reinforcing the similarity between the *C. subternata* extracts.

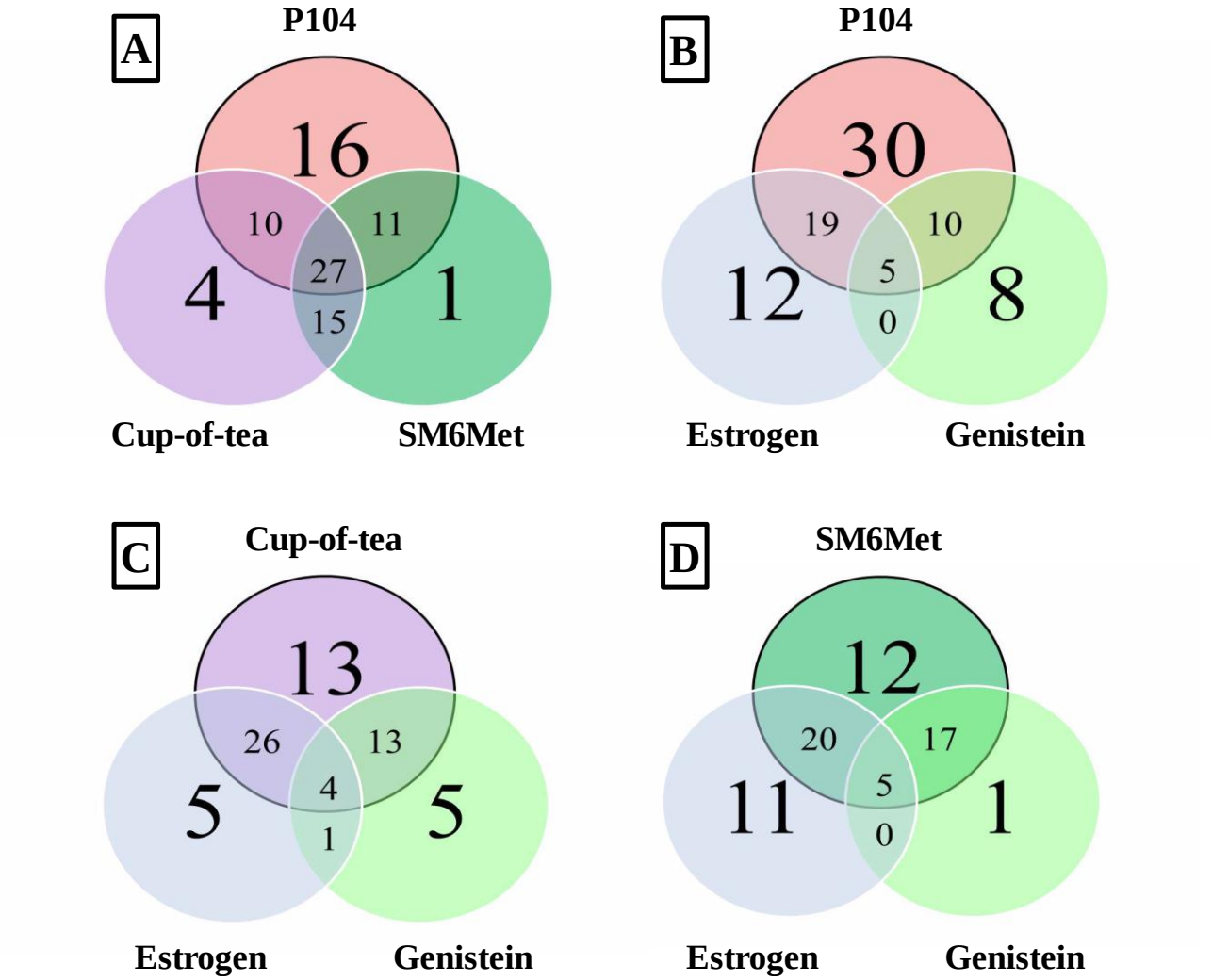

Figure 6. Relative to the group of *Cyclopia* extracts, E_2, and genistein, individual *Cyclopia* extracts regulate distinct and common genes. Venn diagrams, illustrating common and distinct gene regulation, were generated for (A) P104, cup-of-tea, and SM6Met, (B) P104, E_2, and genistein, (C) cup-of-tea, E_2, and genistein, and (D) SM6Met, E_2, and genistein, from fold regulation data obtained from the human breast cancer RT^2 Profiler PCR array (Table S1). Regulation was determined as ± 2 fold change from solvent control. Figures are representative of three independent experiments, except for genistein, which represents one experiment.

Upon comparing gene regulation patterns of P104, E_2, and genistein we found 30, 12, and 8 genes, respectively, to be distinctly regulated within this comparison (Fig 6B). Within this grouping, only 5 genes were commonly regulated by E_2, genistein and P104. Of the 19 genes regulated by P104 and E_2 but not genistein, 89% were regulated in the same direction and 11% in different directions,

reflecting the gene expression patterns observed in Fig. 5. P104 and genistein commonly regulated 10 genes that were not regulated by E_2, of which 80% were regulated in the same direction and 20% in different directions. No genes were regulated by E_2 and genistein but not P104. Of the 5 genes commonly regulated by all three treatments, 60% were regulated in the same direction.

Treatment of MCF-7BUS cells with the cup-of-tea extract, E_2, and genistein (Fig. 6C) resulted in four of the genes being regulated by all three of the treatments of which 50% were regulated in the same direction, with E_2 being the outlier in all of the genes regulated in different directions. Of the 26 genes regulated by the cup-of-tea extract and E_2, but not genistein, only 23% were regulated in different directions. The cup-of-tea extract and genistein, but not E_2, regulated 13 genes, 62% of which were regulated in the same direction and 38% in different directions. Within this comparison only one gene was jointly regulated by E_2 and genistein, but not by cup-of-tea. This gene was regulated in the same direction.

SM6Met, E_2, and genistein commonly regulated 5 genes within this comparison group (Fig. 6D) of which 60% were regulated in the same direction and 40% in different directions. Of the 20 genes co-regulated by SM6Met and E_2, but not genistein, 86% were regulated in the same direction and 14% in different directions. SM6Met and genistein, but not E_2, jointly regulated 17 genes within this group of which 59% were in the same direction and 41% in a different direction. No genes were uniquely shared between genistein and E_2 within this group.

Therefore, when comparing commonly regulated genes, the *Cyclopia* extracts regulated more genes in common with each other than with either E_2 or genistein (Fig. 6A). Specifically, P104 regulated 37 and 38 genes commonly with cup-of-tea and SM6Met, respectively, but only 24 and 15 genes commonly with E_2 and genistein, respectively, while cup-of-tea regulated 42 genes commonly with SM6Met, but only 30 and 17 commonly with E_2 and genistein, respectively, and SM6Met only regulated 26 and 22 genes commonly with E_2 and genistein, respectively. P104 uniquely regulated

the largest number of genes (16 vs. 4 and 1, in Fig. 6A, and 30 vs. 12 and 8, in Fig. 6B) and even within the commonly regulated genes of the group of *Cyclopia* extracts (27 genes) P104 regulated 82% of the jointly regulated genes in the opposite direction to that of cup-of-tea and SM6Met. Furthermore, in only one of the groupings E_2 and genistein jointly regulated a gene that was not regulated by a *Cyclopia* extract and they generally shared more regulated genes with the *Cyclopia* extracts than with each other. Therefore, our results indicate that although the extracts are more similar to each other than to either E_2 or genistein, the extracts from different species regulated genes in different ways and P104 specifically may be considered an outlier within the *Cyclopia* grouping. Furthermore, E_2 and genistein behave more like the *Cyclopia* extracts than each other within the context of the PCR array data.

In addition, to better understand how these changes in gene regulation may impact breast cancer survival and progression, we grouped the genes according to function (Table 3 and Table S2). Concerning survival, growing cancer cells, like normal cells, require nutrients and oxygen and furthermore, they require a system to remove carbon dioxide and metabolic waste [30,38]. The formation of new blood vessels, angiogenesis, is employed by tumours to provide for these requirements [30,38,39]. The extracts of *Cyclopia* regulated more angiogenesis related genes than either E_2 or genistein, however, P104 up-regulated the highest percentage of regulated genes, whilst cup-of-tea and SM6Met, down-regulated a greater percentage of regulated genes. Furthermore, of the 64% angiogenesis related genes up-regulated by P104, 78% promote angiogenesis whereas 22% inhibit angiogenesis and of the 21% down-regulated genes, 67% promote angiogenesis and 33% inhibit angiogenesis. In addition, 50% of the genes up-regulated by cup-of-tea promote angiogenesis, while 50% inhibits angiogenesis and 86% of the genes down-regulated by this extract promotes angiogenesis, while 14% inhibits angiogenesis. All of the genes up-regulated by SM6Met inhibit angiogenesis, while 89% of the down-regulated genes promote angiogenesis and 11% inhibit

angiogenesis. Therefore, not only is there a difference in the gene regulation pattern of the extracts from different species of *Cyclopia*, the extract from *C. genistoides*, P104, seems to be more markedly pro-angiogenic than the extracts from *C. subternata*, cup-of-tea and SM6Met. Furthermore, of the E_2 regulated angiogenic genes, 83% of the total genes regulated were down-regulated of which 80% promote angiogenesis and 20% inhibit angiogenesis and all of the up-regulated genes (17%) inhibit angiogenesis. Therefore, with regards to the promotion of angiogenesis, the P104 extract regulated the majority of the regulated genes towards angiogenesis (67%), whereas E_2, cup-of-tea and SM6Met only regulated 17%, 22%, and 8%, respectively, of the regulated genes towards angiogenesis.

Furthermore, regarding cancer cell invasion, not only is angiogenesis an important contributing factor, but also adhesion, the EMT transition, and proteolysis. Adhesion, specifically, plays an important role in the motility and invasiveness of cells with invasive cells being less adhesive and thus more mobile [49]. Adhesion related genes were regulated similarly by the extracts as for angiogenesis related genes, with P104 up-regulating the majority of regulated genes, while cup-of-tea and SM6Met down-regulated the majority of the regulated genes. During EMT a cell loses its epithelial phenotype, and assumes a mesenchymal phenotype, which allows the cell to migrate from the parent tissue and invade adjacent tissues [50]. The most notable regulator of EMT related genes was the P104 extract, which regulated all of the EMT related genes. A pattern of regulation, similar to that of angiogenesis and adhesion, was also observed regarding proteolysis, a process whereby proteolytic enzymes degrade the extracellular matrix thus increasing cell motility and invasion [40]. Therefore, concerning our results for genes implicated in cancer cell invasion, specifically adhesion, EMT transition, and proteolysis, the P104 extract of *Cyclopia* generally up-regulated a larger number of genes, while cup-of-tea and SM6Met down-regulated a larger number of genes. Specifically, of the cancer invasion genes regulated by each extract, P104 up-regulated 79% of the

regulated genes of which 67% promote invasion whereas 33% inhibit invasion, while down-regulating 21% of the regulated genes of which 75% promote invasion and 25% inhibit invasion. Furthermore, the cup-of-tea extract up-regulated 27% of the regulated genes of which 67% promote invasion and 33% inhibit invasion, while down-regulating 73% of the regulated of which 63% promote invasion and 37% inhibit invasion, while SM6Met up-regulated 24% of the regulated genes of which 50% promote invasion and 50% inhibit invasion, while down-regulating 76% of the regulated genes of which 54% promote invasion and 46% inhibit invasion. Estrogen regulated 32% of the genes that regulate cancer invasion. Of the E_2 regulated genes 71% are up-regulated of which 60% promote invasion and 40% inhibit invasion, while of the 29% down-regulated genes, 50% promote invasion and 50% inhibit invasion. To summarise, when all the genes involved in cancer invasion are considered, whether up- or down-regulated, P104, an extract of *C. genistoides*, regulated 58% of the regulated genes towards promoting cancer invasion, while the cup-of-tea and SM6Met extracts, *C. subternata* extracts, regulated 46% and 47%, respectively, towards promoting invasion. E_2 regulates 57% of the genes towards promoting invasion. Therefore, by evaluating the regulation of invasion associated genes by the *Cyclopia* extracts, although an accurate prediction cannot be made of whether the *Cyclopia* extracts would inhibit or promote cancer cell invasion, it does appear as if P104, like E_2, tends slightly more towards the promotion of cancer invasion.

Damaged DNA can be pro-mutagenic and contribute to cancer development [51]. The basis of cancer development and progression is abnormal cell growth [52], where the normal tightly controlled cycle of growth, division, and apoptosis is disrupted [30,53,54]. Therefore, concerning abnormal cell growth, we will evaluate the regulation by the *Cyclopia* extracts of genes involved in DNA damage, the cell cycle, and apoptosis together.

The previously observed pattern of gene regulation, P104 up-regulated the majority of regulated genes, while cup-of-tea and SM6Met down-regulated the majority of the regulated genes, was also

observed for genes involved in cell growth. P104 up-regulated 86% of the regulated genes with 56% of the up-regulated genes promoting cell growth, while 44% inhibit cell growth and of the 14% down-regulated genes, 25% promote growth and 75% inhibit growth. The cup-of-tea extract, in contrast, only up-regulated 29% of the regulated genes of which 67% promote growth and 33% inhibit growth and furthermore, the extract down-regulated 71% of the regulated genes of which 47% promote growth and 53% inhibit growth. In addition, of the regulated genes, SM6Met up-regulated 29% of which 57% promote growth and 43% inhibit growth, while of the 71% down-regulated genes, 41% promote growth and 59% inhibit growth. Estrogen up-regulated 50% of the regulated genes of which 71% promote growth and 29% inhibit growth and furthermore, of the 50% down-regulated genes, 14% promote growth and 86% inhibit growth. To summarise, when all the genes involved in cancer cell growth are considered, whether up- or down-regulated, E_2, P104, cup-of-tea, and SM6Met, all regulated genes towards cell growth (79%, 59%, 57%, and 58%, respectively), although the extracts of *Cyclopia* did not regulate genes towards cell growth to the same extent as E_2.

Furthermore, the PCR array also allows for the evaluation of genes involved in xenobiotic transport as up-regulation of xenobiotic transport proteins may convey resistance to breast cancer drug treatment [55]) as well as genes of transcription factors, such as the ER subtypes and the progesterone receptor (PR), that may contribute to the development and progression of breast cancer [45,56-59]. Within these functional groups the *Cyclopia* extracts generally regulated a larger number of genes than E_2 and genistein, with P104 up-regulating the majority of regulated genes and the cup-of-tea and SM6Met extracts down-regulating the majority of regulated genes.

To conclude, the *Cyclopia* extracts regulated the expression of genes that are known to play a role in breast cancer. Furthermore, not only did the extracts regulate distinct as well as common genes, when compared to E_2 and genistein, the extracts also regulated distinct and common genes when

compared to each other. Also, of the genes that are commonly regulated by the *Cyclopia* extracts, P104, a *C. genistoides* extract, generally regulated these genes in a different direction from that of SM6Met and cup-of-tea, *C. subternata* extracts.

5.4.4. Discussion

Upon cancer initiation a cell acquires capabilities, referred to as hallmark capabilities, which may allow the initiated cancer cell to sustain proliferative signalling, evade growth suppressors, resist cell death, enable replicative immortality, induce angiogenesis, and activate invasion and metastasis [30]. In this chapter we focussed on hallmark capabilities that allow for the growth, by sustaining proliferative signalling and resisting cell death, and survival, by promoting angiogenesis, of the cancer cell, as well as those that promote invasion and metastasis.

During initiation genomic DNA damage may occur and this may lead to gene mutations that change the characteristics of a cell Some of these gene mutations may convey survival and growth advantages to the cancer cell by altering the expression levels of genes coding for the proteins associated with these processes and furthermore, during progression, gene mutations may increase the invasive and metastatic potential of the cells [4-6,16,17,36,37]. Furthermore, exogenous signals, such as E_2 in breast cancer, may exacerbate these processes by also activating mutagenic, growth promoting or proliferation factors. Therefore, using the Human Breast Cancer RT^2 Profiler™ PCR Array, we evaluated the effect of the *Cyclopia* extracts on the expression of genes associated with the cell cycle and apoptosis, as well as genes that may influence cancer cell survival and invasion via angiogenesis, EMT, adhesion and proteolysis. We found that the *Cyclopia* extracts generated global gene expression profiles that were different from that of E_2 although, the extract from *C. genistoides*, P104, like E_2, generally increased the expression levels of the regulated genes, whereas, cup-of-tea and SM6Met, *C. subternata* extracts, unlike E_2 and P104, generally decreased the expression levels of regulated genes (Fig. 5). Upon comparison of the regulation patterns of the

Cyclopia extracts we found that all of the extracts regulated both common and distinct genes (Fig. 6A), with 32% of the genes regulated by all three extracts. Furthermore, closer inspection of these commonly regulated genes revealed that P104 regulated 82% of these genes in a different direction than cup-of-tea and SM6Met, implying interspecies differences in the modulation of breast cancer associated genes by *Cyclopia* extracts. Furthermore, upon comparison of the regulation patterns of each of the individual extracts grouped with E_2 and genistein (Figs. 6B-D), we found that, generally all of the extracts regulated more genes in common with E_2 than with genistein, suggesting that, concerning the global number of genes regulated, the extracts behaved more like E_2 than genistein. In addition, we functionally grouped all of the genes to evaluate how afore mentioned changes in gene expression would affect the cell phenotype (Table 3). Concerning genes that affect angiogenesis, we found that P104 generally regulated genes towards the promotion of angiogenesis whereas E_2, cup-of-tea, and SM6Met regulated genes towards the inhibition of angiogenesis. Our results for angiogenesis with the cup-of-tea and SM6Met extracts are promising as inhibition of angiogenesis would inhibit the growth and survival of cancer tumours, but as our results for E_2 are in conflict with the known role of E_2 in angiogenesis [60], further research, using both *in vitro* [61] as well as *in vivo* [62] angiogenesis models, into these findings is warranted.

In addition, the array allows for the evaluation of the expression levels of genes that would alter the growth of cells. We found that E_2 regulated genes towards cell growth and that the extracts, like E_2, also regulated the majority of genes towards cell growth, although the number of genes regulated towards growth was lower than that of E_2. These findings suggest that the extracts of *Cyclopia*, like E_2, may promote cell growth although not to the same extent. Previously (Chapter 3), using the MCF-7BUS cell proliferation assay we found that although the *Cyclopia* extracts did indeed induce cell proliferation it was not to the same extent as E_2, in accordance with our PCR array findings concerning the number of genes regulated towards growth promotion. Furthermore, the

proliferation marker Ki-67 was more robustly up-regulated by E_2 than by any of the *Cyclopia* extracts. In addition, we also found that in the presence of E_2, the *Cyclopia* extracts antagonised E_2-induced cell proliferation. This prompted us to evaluate changes in MCF-7BUS cell distribution between the phases of the cell cycle after treatment with *Cyclopia* extracts, as progression of cells through the cell cycle is a coordinated process consisting of several phases that allows for the regulation of cell growth [8,9,18,19] and thus, perturbation of cell distribution between the phases of the cycle may provide insight into how the *Cyclopia* extracts modulate MCF-7BUS cell proliferation. Upon E_2 induction the number of cells in the G0/G1 phase (checkpoint for DNA damage) decreased and the number of cells in the S phase (DNA synthesis) and G2/M phase (duplicated DNA checked for damage and cells progress to mitosis) increased. Similar distribution patterns after E_2 treatment have previously been reported and this distribution pattern is assigned to E_2 induced proliferation [63-65]. A similar distribution pattern was observed for genistein, albeit less pronounced, and this phase distribution following genistein treatment has also been previously shown for low concentrations (0.1- 10μM) of genistein [66]. Interestingly, treatment of cells with luteolin, enterodiol, coumestrol, and *Cyclopia* extracts uniformly increased the number of cells in the S phase, while decreasing the number of cells in the G0/G1 and G2/M phases. This distribution has previously been shown for coumestrol [66] and the increase in the S phase, also seen for E_2 and genistein, is suggested to be the result of the stimulation of S phase activity with resultant proliferation. However, the cell cycle may also be disrupted by arresting cells in the S phase due to DNA not being replicated [67]. As a possible scenario, this arrest may be achieved by the inhibition of cyclin-dependent kinase 2 (CDK2) as CDK2 inhibition prevents the initiation of DNA synthesis [9]. The cup-of-tea and SM6Met extracts both down-regulated CDK2 expression levels, while P104 up-regulated the levels of ataxia telangiectasia mutated (ATM), an inhibitor of CDK2 [9] (Table S1 & S2). Therefore, we may postulate that the extracts of *Cyclopia* could be disrupting

the cell cycle by arresting cells in the S phase through either down-regulating CDK2 or by up-regulating the inhibitor of CDK2, ATM. However, further experiments are required to confirm whether this is indeed an S phase arrest or whether the cells were analysed at a stage where they were on the brink of G2/M phase entry. To resolve this issue, for future studies the cell cycle distribution assays should be repeated with the added modification of synchronizing the cells in the G0/G1 phase via serum starvation before induction [68,69]. Furthermore, the PCR array findings for CDK2 and ATM gene expression levels should be validated as well as the effect of *Cyclopia* extracts on CDK2 and ATM protein levels. Furthermore, treatment of MCF-7BUS cell with E_2 and *Cyclopia* extracts resulted in the up-regulation of the mRNA levels (Table S1) of promoters of G0/G1 to S phase progression (cyclins D1, D2, & especially E1) [67] and the down-regulation of cyclin dependent kinase inhibitor 2A (CDKN2A), a negative regulator of G1/S transition cyclins, as well as cell proliferation [70,71]. Therefore, in conclusion, we suggest that in the absence of E_2, cell proliferation is induced by the *Cyclopia* extracts, as well as E_2, by the stimulation of G1 phase to S phase progression after which the cell is committed to complete the cycle [15], but that in the case of the *Cyclopia* extracts, unlike E_2, DNA synthesis is not fully completed and thus a S phase arrest results.

Furthermore, in an attempt to elucidate the mechanism whereby *Cyclopia* extracts inhibited E_2-induced MCF-7BUS cell proliferation (Chapter 3), we evaluated changes in cell cycle phase distribution after treatment of MCF-7BUS cells with *Cyclopia* extracts in the presence of E_2. We found no significant deviation from E_2-induced phase distribution with the polyphenols, luteolin, coumestrol, and the *Cyclopia* extracts, P104 and cup-of-tea, except for slight increases in the percentage apoptotic cells. The polyphenols, genistein and enterodiol, however, increased the percentage of cells in the G2/M phase of the cell cycle. For genistein, it has been shown that after a 72 hour treatment of MCF-7 cells with 20 μg/ml genistein, proliferation was inhibited by inducing a

G2/M arrest [72], therefore, in the presence of E_2, we may assume that cell proliferation is inhibited by genistein and enterodiol by inducing a G2/M phase arrest. Interestingly, the *Cyclopia* extract, SM6Met, induced a significant G0/G1 phase arrest in the presence E_2, similar to the SERM, tamoxifen [73], which has protective properties in breast tissue. Therefore we postulate that MCF-7BUS cell proliferation, in the presence of E_2, is inhibited by the polyphenols, genistein and enterodiol, by inducing cell cycle arrest in the G2/M phase and by the *Cyclopia* extract, SM6Met, by inducing a G0/G1 phase arrest in the cell cycle, thus disrupting the cell cycle distribution pattern assigned to E_2-induced proliferation. For future studies, as for in the absence of E_2, the cell cycle distribution assays may be repeated with the added modification of synchronizing the cells in the G0/G1 phase via serum starvation before induction [68,69]. This modification may provide more significant distribution changes and potentially provide helpful information on the modulation of MCF-7BUS cell proliferation by the *Cyclopia* extracts, P104 and cup-of-tea, in the presence of E_2. In addition, the PCR array may be repeated with MCF-7BUS cells treated with the *Cyclopia* extracts in the presence of E_2 to evaluate how this would modulate the expression levels of genes associated with cell growth to establish whether differences in gene expression may explain the antagonism of E_2-induced cell proliferation by the *Cyclopia* extracts, specifically evaluating apoptosis associated gene expression as well as genes of proteins that regulate progression from the G0/G1 phase to the S phase of the cell cycle.

Furthermore, using the PCR array, we evaluated genes that have been linked to cancer cell invasion and found that all of the extracts, as well as E_2, regulated genes in such a way that it is difficult to predict how these treatments would affect the invasive phenotype of the MCF-7BUS cells. However, these findings may help to interpret our findings with the MCF-7BUS cell invasion assay (Fig. 4). Using this assay we found that E_2 did not affect the number of invasive MCF-7BUS cells and that none of the polyphenols or *Cyclopia* extracts significantly affected the number of invasive

cells. Therefore, we may postulate that, treatment with E_2 and the *Cyclopia* extracts do not increase the invasive capabilities of the MCF-7BUS cells and that the invasion associated genes were regulated in such a way that the net regulation of all the genes did not result in a phenotypic shift to either increased or decreased invasive capabilities. Interestingly, although having no effect in the absence of E_2, we found that in the presence of E_2, like cells treated with the ER antagonist, ICI 182,780, the number of invasive cells was significantly increased by the *Cyclopia* extracts, P104 and SM6Met. Previously, Goto *et al.* [74] also reported an increase in the number of invasive cells following treatment with ICI 182,780. As we did not evaluate the effect of the *Cyclopia* extracts on gene expression in the presence of E_2, we cannot ascribe changes in gene expression levels to our invasion assay findings in the presence of E_2. Therefore, for future studies, it may be beneficial to evaluate the modulation of gene expression by the *Cyclopia* extracts in the presence of E_2 and furthermore, not only to evaluate gene expression and invasive capabilities of the MCF-7BUS cells, but also that of a highly invasive cell line such as the MCF-7-M5 cell line [74].

To conclude, our findings provide insight into how *Cyclopia* extracts regulate processes involved in the promotion and progression of breast cancer. The *Cyclopia* extracts may sustain proliferative signalling by up-regulating genes that promote cell growth, although not to the same extent than E_2. Furthermore, the extracts of *C. subternata*, cup-of-tea and SM6Met, up-regulated genes that inhibit angiogenesis, which is beneficial as it may inhibit the survival of cancer tumours. In addition, our findings concerning cell invasion, showing that the ER antagonist increased the number of invasive cells, supports the finding that the ER may have protective properties during the later stages of tumour progression [74]. Furthermore, our MCF-7BUS cell cycle assay results provide insight into the mechanism whereby the *Cyclopia* extracts modulate MCF-7BUS cell proliferation. Finally, our findings show that although all of the extracts of *Cyclopia* may promote cell growth, the extracts from different species have different mechanisms of doing so. For future studies, the PCR array

may be repeated to evaluate gene regulation by the *Cyclopia* extracts in the presence of E_2 to obtain further insight into the mechanism whereby the extracts antagonises E_2-induced MCF-7BUS cell proliferation. In addition, the PCR array results should be validated using quantitative-PCR and furthermore, the findings regarding angiogenesis associated genes should be followed up by both *in vitro* as well as *in vivo* angiogenesis test models.

5.5. Literature cited

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5.6. Supporting information

Table S1. Alphabetical list of proteins, involved in breast cancer development and progression, regulated by E_2, genistein, and the *Cyclopia* extracts.

	Fold change from solvent treated cells				
Protein name	**10^{-9}M E_2**	**10^{-9}M Genistein**	**9.8µg/ml P104**	**9.8 µg/ml Cup-of-tea**	**9.8 µg/ml SM6Met**
ATP-binding cassette, sub-family B (MDR/TAP), member 1	1.67	-3.15[a]	1.45	-3.23	-2.59
ATP-binding cassette, sub-family G (WHITE), member 2	1.43	-1.36	3.05[b]	-1.09	-1.83
ADAM metallopeptidase domain 23	1.39	1.15	2.55	-4.61	-12.46
V-akt murine thymoma viral oncogene homolog 1	-46.38	-1.21	-1.48	-2.17	-45.42
Adenomatous polyposis coli	1.57	1.13	3.77	-1.19	-3.80
Androgen receptor	1.33	-1.34	3.14	1.60	1.12
Ataxia telangiectasia mutated	1.71	1.86	4.31	-1.89	-1.43
BCL2-associated agonist of cell death	1.29	1.25	2.79	1.59	-1.21
B-cell CLL/lymphoma 2	1.53	-1.11	30.99	1.92	1.18
Baculoviral IAP repeat containing 5	6.27	100.01	14.73	6.13	2.97
Breast cancer 1, early onset	1.34	-1.13	2.99	1.74	-1.33
Breast cancer 2, early onset	1.36	-1.44	3.11	1.40	-1.47
Cyclin A1	1.25	1.43	5.13	-1.35	-2.54
Cyclin D1	1.77	1.73	123.57	4.49	1.64
Cyclin D2	1.20	-1.59	2.53	-14.37	-12.97
Cyclin E1	74.72	1.26	10.51	10.84	2.29
Cadherin 1, type 1, E-cadherin (epithelial)	-2.42	1.16	-1.02	1.04	87.52
Cadherin 13, H-cadherin (heart)	-1.24	-1.64	2.45	-1.05	-5.03
Cyclin-dependent kinase 2	1.44	1.10	4.95	-3.69	-5.17
Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	4.20	-1.05	2.58	2.85	1.96

Cyclin-dependent kinase inhibitor 1C (p57, Kip2)	-3.02	-5.38	-9.20	-89.85	-668.50
Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	1.20	21.22	1.57	-16.59	-22.68
Colony stimulating factor 1 (macrophage)	3.14	1.33	-191.79	-968.30	-1695.11
Cystatin E/M	1.29	3.53	3.21	-6.55	-3.92
Catenin (cadherin-associated protein), beta 1, 88kDa	-8.50	1.16	-2.60	-5.09	-9.69
Cathepsin D	63.22	1.22	3.18	5.04	107.59
Epidermal growth factor	-2.40	-1.09	-1.11	-2.87	-9.61
Epidermal growth factor receptor	1.84	2.69	6.70	-1.85	-2.29
V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	1.27	-1.03	2.54	-1.01	-1.75
Estrogen receptor 1	11.57	-1.02	7.44	1.93	1.22
Estrogen receptor 2 (ER beta)	1.81	11.36	40.67	-3.26	2.79
Forkhead box A1	1.26	-1.20	1080.73	1.88	1.27
GATA binding protein 3	273.41	-1.86	41.47	41.64	1.24
GLI family zinc finger 1	1.03	-5.38	1.34	-18.61	-2.94
Growth factor receptor-bound protein 7	-2.37	-1.24	-1.26	-4.02	-1.90
Glutathione S-transferase pi 1	-1.47	-29.09	-1.12	-8.88	-30.85
Hypermethylated in cancer 1	2.89	-5.38	5.87	-2.40	-2.90
Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	-3.97	1.16	-1.71	2.01	-4.03
Insulin-like growth factor 1 (somatomedin C)	1.20	-5.38	1.57	-16.59	-22.68
Insulin-like growth factor 1 receptor	12.91	1.34	3.58	2.42	1.56
Insulin-like growth factor binding protein 3	4.30	-2.58	2.61	-2.30	-2.97
Interleukin 6 (interferon, beta 2)	1.60	2.39	-1.94	-15.82	-2.95
Jun proto-oncogene	-7.35	-1.01	9.31	1.52	-3.51
Keratin 18	-2.43	-1.01	-1.35	3.13	2.29

Keratin 19	-2.63	1.55	-1.01	-3.58	-2.63
Keratin 5	1.20	-5.38	11.62	-16.59	-22.68
Keratin 8	1.35	1.54	3.47	9.08	22.13
Mitogen-activated protein kinase 1	1.30	-1.49	2.81	2.42	1.59
Mitogen-activated protein kinase 3	9.37	1.38	22.06	-1.37	1.79
Mitogen-activated protein kinase 8	1.36	-1.13	2.72	1.38	-1.18
O-6-methylguanine-DNA methyltransferase	22.01	1.17	2.66	2.34	1.46
Antigen identified by monoclonal antibody Ki-67	15.31	-1.15	2.29	1.28	1.91
MutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli)	1.44	1.09	101.41	1.63	15.41
Matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	1.20	-5.38	1.57	-16.59	-22.68
Matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	1.23	13.36	2.84	-1.52	-6.51
Mucin 1, cell surface associated	10.14	8.71	4.36	1.37	5.00
V-myc myelocytomatosis viral oncogene homolog (avian)	1.95	1.67	1.10	-5.23	1.70
Non-metastatic cells 1, protein (NM23A) expressed in	-2.12	-1.02	-1.48	-2.11	-1.30
Notch 1	1.41	6.90	4.69	-1.08	2.47
Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	-44.13	-1.05	-14.01	-55.10	-86.23
Progesterone receptor	1.91	1.77	-1.17	-4.27	-1.27
Plasminogen activator, urokinase	1.13	-1.23	7.47	-3.20	-5.68
PR domain containing 2, with ZNF domain	1.35	1.02	3.16	2.98	-1.53
Phosphatase and tensin homolog	-5.37	-1.26	-2.50	-2.64	-3.50
Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	1.20	-5.38	1.57	-16.59	-13.50
PYD and CARD domain containing	1.24	1.42	-7.23	-4.29	-14.71
Retinoic acid receptor, beta	1.04	3.39	3.58	-2.33	-7.04

Ras association (RalGDS/AF-6) domain family member 1	-3.88	1.08	-1.69	-5.78	-12.00
Retinoblastoma 1	1.33	1.18	3.27	1.63	1.17
Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	1.36	1.83	3.11	-2.20	-2.94
Stratifin	3.27	1.40	2.99	3.12	1.72
Secreted frizzled-related protein 1	1.20	-5.38	1.57	-16.59	-22.68
Solute carrier family 39 (zinc transporter), member 6	2.34	-1.10	2.23	-5.14	2.07
Slit homolog 2 (Drosophila)	1.16	-1.54	3.62	-2.91	1.09
Snail homolog 2 (Drosophila)	-2.95	1.13	-1.08	-13.05	-21.61
V-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)	1.25	-1.08	30.87	6.17	-1.68
Trefoil factor 3 (intestinal)	1.82	1.37	3.14	1.65	-1.05
Transforming growth factor, beta 1	23.10	-1.09	2.89	1.35	-1.08
Thrombospondin 1	31.34	1.85	4.65	4.65	90.60
Tumor protein p53	-137.15	-1.00	-63.16	-57.88	-175.28
Tumor protein p73	1.16	9.70	3.39	-1.50	-2.96
Twist homolog 1 (Drosophila)	1.14	1.04	2.44	1.00	-1.68
Vascular endothelial growth factor A	1.53	2.39	3.76	1.27	1.08
X-box binding protein 1	1.60	1.49	3.81	6.66	3.91

[a]Green denotes > 2 fold down-regulation compared to solvent treated cells.
[b]Red denotes >2 fold up-regulation compared to solvent treated cells.

Table S2. Functional grouping of proteins, involved in breast cancer development and progression regulated, by E_2, genistein, and the *Cyclopia* extracts.

		Fold change from solvent control treated cells				
Functional group	**Protein name**	**10^{-9}M E_2**	**10^{-9}M Genistein**	**9.8µg/ml P104**	**9.8µg/ml Cup-of-tea**	**9.8µg/ml SM6Met**
Angiogenesis	Cadherin 13, H-cadherin (heart)	-1.24	-1.64	2.45[a]	-1.05	-5.03[b]
	Catenin (cadherin-associated protein), beta 1, 88kDa	-8.50	1.16	-2.60	-5.09	-9.69
	Epidermal growth factor	-2.40	-1.09	-1.11	-2.87	-9.61
	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	1.27	-1.03	2.54	-1.01	-1.75
	Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	-3.97	1.16	-1.71	2.01	-4.03
	Interleukin 6 (interferon, beta 2)	1.60	2.39	-1.94	-15.82	-2.95
	Jun proto-oncogene	-7.35	-1.01	9.31	1.52	-3.51
	Notch 1	1.41	6.90	4.69	-1.08	2.47
	Plasminogen activator, urokinase	1.13	-1.23	7.47	-3.20	-5.68
	Phosphatase and tensin homolog	-5.37	-1.26	-2.50	-2.64	-3.50
	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	1.36	1.83	3.11	-2.20	-2.94
	Slit homolog 2 (Drosophila)	1.16	-1.54	3.62	-2.91	1.09
	Thrombospondin 1	31.34	1.85	4.65	4.65	90.60
	Vascular endothelial growth factor A	1.53	2.39	3.76	1.27	1.08
Epithelial to mesenchymal transition	Catenin (cadherin-associated protein), beta 1, 88kDa	-8.50	1.16	-2.60	-5.09	-9.69
	Notch 1	1.41	6.90	4.69	-1.08	2.47
	V-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)	1.25	-1.08	30.87	6.17	-1.68
	Transforming growth factor, beta 1	23.10	-1.09	2.89	1.35	-1.08
	Twist homolog 1 (Drosophila)	1.14	1.04	2.44	1.00	-1.68
Adhesion	ADAM metallopeptidase domain 23	1.39	1.15	2.55	-4.61	-12.46

	Adenomatous polyposis coli	1.57	1.13	3.77	-1.19	-3.80
	B-cell CLL/lymphoma 2	1.53	-1.11	30.99	1.92	1.18
	Cadherin 1, type 1, E-cadherin (epithelial)	-2.42	1.16	-1.02	1.04	87.52
	Cadherin 13, H-cadherin (heart)	-1.24	-1.64	2.45	-1.05	-5.03
	Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	1.20	21.22	1.57	-16.59	-22.68
	Colony stimulating factor 1 (macrophage)	3.14	1.33	-191.79	-968.30	-1695.11
	Catenin (cadherin-associated protein), beta 1, 88kDa	-8.50	1.16	-2.60	-5.09	-9.69
	Epidermal growth factor receptor	1.84	2.69	6.70	-1.85	-2.29
	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	1.27	-1.03	2.54	-1.01	-1.75
	Phosphatase and tensin homolog	-5.37	-1.26	-2.50	-2.64	-3.50
	Transforming growth factor, beta 1	23.10	-1.09	2.89	1.35	-1.08
	Thrombospondin 1	31.34	1.85	4.65	4.65	90.60
Proteolysis	ADAM metallopeptidase domain 23	1.39	1.15	2.55	-4.61	-12.46
	Cystatin E/M	1.29	3.53	3.21	-6.55	-3.92
	Cathepsin D	63.22	1.22	3.18	5.04	107.59
	Matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	1.20	-5.38	1.57	-16.59	-22.68
	Matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	1.23	13.36	2.84	-1.52	-6.51
	Plasminogen activator, urokinase	1.13	-1.23	7.47	-3.20	-5.68
	PYD and CARD domain containing	1.24	1.42	-7.23	-4.29	-14.71
Apoptosis	V-akt murine thymoma viral oncogene homolog 1	1.39	1.15	2.55	-4.61	-12.46
	Adenomatous polyposis coli	1.57	1.13	3.77	-1.19	-3.80
	BCL2-associated agonist of cell death	1.29	1.25	2.79	1.59	-1.21
	B-cell CLL/lymphoma 2	1.53	-1.11	30.99	1.92	1.18
	Cadherin 1, type 1, E-cadherin (epithelial)	-2.42	1.16	-1.02	1.04	87.52
	Cadherin 13, H-cadherin (heart)	-1.24	-1.64	2.45	-1.05	-5.03

	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	4.20	-1.05	2.58	2.85	1.96
	Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	1.20	21.22	1.57	-16.59	-22.68
	Glutathione S-transferase pi 1	-1.47	-29.09	-1.12	-8.88	-30.85
	Insulin-like growth factor 1 (somatomedin C)	1.20	-5.38	1.57	-16.59	-22.68
	Interleukin 6 (interferon, beta 2)	1.60	2.39	-1.94	-15.82	-2.95
	Jun proto-oncogene	-7.35	-1.01	9.31	1.52	-3.51
	Mucin 1, cell surface associated	10.14	8.71	4.36	1.37	5.00
	Non-metastatic cells 1, protein (NM23A) expressed in	-2.12	-1.02	-1.48	-2.11	-1.30
	Retinoic acid receptor, beta	1.04	3.39	3.58	-2.33	-7.04
	Stratifin	3.27	1.40	2.99	3.12	1.72
	Secreted frizzled-related protein 1	1.20	-5.38	1.57	-16.59	-22.68
	Tumor protein p53	-137.15	-1.00	-63.16	-57.88	-175.28
	Tumor protein p73	1.16	9.70	3.39	-1.50	-2.96
	Twist homolog 1 (Drosophila)	1.14	1.04	2.44	1.00	-1.68
Cell cycle	Adenomatous polyposis coli	1.57	1.13	3.77	-1.19	-3.80
	B-cell CLL/lymphoma 2	1.53	-1.11	30.99	1.92	1.18
	Cyclin A1	1.25	1.43	5.13	-1.35	-2.54
	Cyclin D1	1.77	1.73	123.57	4.49	1.64
	Cyclin D2	1.20	-1.59	2.53	-14.37	-12.97
	Cyclin E1	74.72	1.26	10.51	10.84	2.29
	Cyclin-dependent kinase 2	1.44	1.10	4.95	-3.69	-5.17
	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	4.20	-1.05	2.58	2.85	1.96
	Cyclin-dependent kinase inhibitor 1C (p57, Kip2)	-3.02	-5.38	-9.20	-89.85	-668.50
	Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	1.20	21.22	1.57	-16.59	-22.68
	Jun proto-oncogene	-7.35	-1.01	9.31	1.52	-3.51
	Antigen identified by monoclonal antibody Ki-67	15.31	-1.15	2.29	1.28	1.91
	V-myc myelocytomatosis viral oncogene homolog (avian)	1.95	1.67	1.10	-5.23	1.70

	Phosphatase and tensin homolog	-5.37	-1.26	-2.50	-2.64	-3.50
	Ras association (RalGDS/AF-6) domain family member 1	-3.88	1.08	-1.69	-5.78	-12.00
	Retinoblastoma 1	1.33	1.18	3.27	1.63	1.17
	Stratifin	3.27	1.40	2.99	3.12	1.72
	Tumor protein p53	-137.15	-1.00	-63.16	-57.88	-175.28
DNA damage	Adenomatous polyposis coli	1.57	1.13	3.77	-1.19	-3.80
	Ataxia telangiectasia mutated	1.71	1.86	4.31	-1.89	-1.43
	Breast cancer 1, early onset	1.34	-1.13	2.99	1.74	-1.33
	Breast cancer 2, early onset	1.36	-1.44	3.11	1.40	-1.47
	Cyclin D1	1.77	1.73	123.57	4.49	1.64
	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	4.20	-1.05	2.58	2.85	1.96
	Mitogen-activated protein kinase 1	1.30	-1.49	2.81	2.42	1.59
	O-6-methylguanine-DNA methyltransferase	22.01	1.17	2.66	2.34	1.46
	MutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli)	1.44	1.09	101.41	1.63	15.41
	Stratifin	3.27	1.40	2.99	3.12	1.72
	Tumor protein p53	-137.15	-1.00	-63.16	-57.88	-175.28
	Tumor protein p73	1.16	9.70	3.39	-1.50	-2.96
Xenobiotic transport	ATP-binding cassette, sub-family B (MDR/TAP), member 1	1.67	-3.15	1.45	-3.23	-2.59
	ATP-binding cassette, sub-family G (WHITE), member 2	1.43	-1.36	3.05	-1.09	-1.83
Transcription factors	Androgen receptor	1.33	-1.34	3.14	1.60	1.12
	Catenin (cadherin-associated protein), beta 1, 88kDa	-8.50	1.16	-2.60	-5.09	-9.69
	Estrogen receptor 1	11.57	-1.02	7.44	1.93	1.22
	Estrogen receptor 2 (ER beta)	1.81	11.36	40.67	-3.26	2.79
	Forkhead box A1	1.26	-1.20	1080.73	1.88	1.27
	GATA binding protein 3	273.41	-1.86	41.47	41.64	1.24
	Hypermethylated in cancer 1	2.89	-5.38	5.87	-2.40	-2.90
	Jun proto-oncogene	-7.35	-1.01	9.31	1.52	-3.51

	V-myc myelocytomatosis viral oncogene homolog (avian)	1.95	1.67	1.10	-5.23	1.70
	Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	-44.13	-1.05	-14.01	-55.10	-86.23
	Progesterone receptor	1.91	1.77	-1.17	-4.27	-1.27
	PR domain containing 2, with ZNF domain	1.35	1.02	3.16	2.98	-1.53
	Retinoic acid receptor, beta	1.04	3.39	3.58	-2.33	-7.04
	Retinoblastoma 1	1.33	1.18	3.27	1.63	1.17
	Tumor protein p53	-137.15	-1.00	-63.16	-57.88	-175.28
	Tumor protein p73	1.16	9.70	3.39	-1.50	-2.96
	X-box binding protein 1	1.60	1.49	3.81	6.66	3.91

[a]Red denotes > 2 fold up-regulation compared to solvent treated cells.
[b]Green denotes >2 fold down-regulation compared to solvent treated cells.

Chapter 6

Final discussion and conclusions

Excessive levels of estrogens can give rise to abnormal cell growth, the basis of cancer development and progression, in estrogen sensitive tissues like the breast [1]. High levels of estrogen not only induce cell hyper-proliferation [2,3], which itself provides an opportunity for DNA damage, but can serve as the chemical carcinogen inflicting DNA damage [4-11]. Furthermore, damaged DNA may initiate the process of cancer development [12,13].

Breast cancer is a global problem [14-16] and there is a need to find effective treatments to either prevent the initiation of cancer or inhibit the progression thereof. Current treatments, at the molecular level, target estrogen production via AIs or ovarian function suppressors [17-22], or estrogen signalling through its cognate receptors via SERMs and SERDs [23-27]. However, although excessive estrogen signalling can be detrimental to an individual's health, abolishment of its function can also have severe consequences. Blocking of estrogen signalling can induce menopause associated side-effects [28-31] as well as a surge in inflammatory diseases [32,33] and an increase in the occurrence of osteoporosis [17,28,34]. Therefore, although current treatments are effective, there is a need for therapeutics with a reduced side-effect profile. As the ER has two subtypes, ERα and ERβ [35,36], and, physiologically, ERα is associated with the promotion of cell proliferation that contributes to the occurrence of breast and endometrial cancer, whereas several studies have shown that ERβ inhibits ERα-dependent cell proliferation and could prevent cancer development [37-45], it has been suggested that SERSMs may offer a safer alternative in breast cancer treatment. Specifically, it has been postulated that finding a treatment that would antagonize ERα [46], while being an ERβ agonist [32,44,45,47], would be an effective treatment to either prevent the initiation of cancer or inhibit the progression thereof at a molecular level, while producing fewer side-effects. The *R*, *R* enantiomer of 5,11-*cis*-diethyl-5,6,11,12-tetrahydrochrysene-2,8-diol (THC) is an ERα agonist and potently antagonizes E_2 function via ERβ [48], which provides a proof of concept that ER subtype specific agonist and antagonist activity

may be found within one compound and thus supports the possibility of finding a compound that behaves inversely. Furthermore, as breast cancer occurs more frequently in postmenopausal women [49,50], a group that often requires HRT, it has been suggested that an ideal HRT would offer a treatment effectively addressing menopausal symptoms, while as a side-effect, preventing breast cancer [51,52]. For this too, SERSMs may present a worthwhile avenue to pursue.

Cyclopia, an indigenous South African plant used to prepare honeybush tea, contains phytoestrogens [53] and is being considered for the preparation of an estrogenic nutraceutical for the treatment of menopausal symptoms. The current work is part of this larger project, but has focussed on evaluating the modulation of molecular targets involved in the prevention and treatment of breast cancer. Specifically, we looked towards *Cyclopia* extracts for finding the elusive ideal SERSM. Previously it has been shown that *Cyclopia* extracts can bind to both ER subtypes [53-55], but can only activate transcription through the ERβ subtype [54,56]. This raised the question that if *Cyclopia* extracts can bind to ERα, but not act as an agonist, can it antagonize E_2 function via ERα? To answer this question we evaluated the SERSM behaviour of *Cyclopia* extracts in COS-1 cells transfected with either ERα or ERβ (Chapter 3) and found that, like previous findings, the methanol extracts of *Cyclopia* acted as ERβ agonists [54,56]. In addition, for the first time we showed that all of the tested *Cyclopia* extracts (P104 [54,55] and SM6Met [56], methanol extracts of *C. genistoides* and *C. subternata*, respectively, and cup-of-tea [56], a water extract of *C. subternata*) antagonized E_2-induced activation of an ERE-containing promoter reporter construct via ERα. In addition, we evaluated the SERSM behaviour of the *Cyclopia* extracts in a breast carcinoma cell line, MCF-7BUS, not only because the breast carcinoma cell line would be relevant to a study of molecular targets involved in breast cancer, but also because it represents a more complex environment where both of the ER subtypes are co-expressed. In this test system we found that all of the *Cyclopia* extracts behaved as agonists by activating transcription of an ERE-

containing promoter reporter construct and concluded that this activation is probably being mediated via ERβ, as we had established, in the model where the subtypes were expressed separately, that the extracts are ERα antagonists and ERβ agonists. In a transrepression model, however, we found that when the ER subtypes were expressed separately, P104, a *C. genistoides* extract, behaved as an ER agonist whereas SM6Met, a methanol extract of *C. subternata*, displayed antagonism towards ERα, in the absence of E_2, and towards ERβ, in the presence of E_2, while the water extract of *C. subternata*, cup-of-tea, displayed ERβ antagonism. Furthermore, in the more complex milieu where both subtypes are co-expressed (MCF-7BUS cells) all of the *Cyclopia* extracts acted as agonists while the water extract of *C. subternata* also displayed ER antagonism. Although these findings do not uniformly support our previous findings in a transactivation model showing ERα antagonism and ERβ agonism, they do show that in a system where the ER subtypes are co-expressed (MCF-7BUS cells), as for our transactivation model, the *Cyclopia* extracts display ER agonist behaviour.

Having shown that *Cyclopia* extracts display SERSM activity on a transcriptional level we investigated whether effects on ER subtype protein levels could offer additional explanations for the observed SERSM activity. We found that, in MCF-7BUS cells, the methanol extracts, like E_2, significantly down-regulated ERα protein levels while, unlike E_2, all extracts significantly increased ERβ protein levels (Chapter 4). Furthermore, in the presence of E_2, the extracts of *Cyclopia* down-regulated ERα protein levels even further than E_2 alone. These findings, in addition to strengthening our argument that the transcriptional effects (transactivation and transrepression) observed in MCF-7BUS cells is probably being mediated via ERβ, also supports a postulate that the observed ERα antagonist behaviour of the *Cyclopia* extracts in the transactivation model, in COS-1 cells, may be due to the down-regulation of ERα levels, as fulvestrant, an ER antagonist, inhibits E_2 signalling through the ER by accelerating the degradation of the ER protein [57-59]. However, as

E_2, an ERα agonist, also down-regulates ERα protein levels [60,61], as a part of the endocrine feedback loop [60], it must be stressed that this is mere speculation and that other avenues must be explored to elucidate how the extracts of *Cyclopia* antagonize E_2-induced activation of an ERE-containing promoter reporter construct via ERα.

The localization to and distribution of ER in the nucleus of a cell may be one of these avenues. Upon ligand activation, both of the ER subtypes localize to the nucleus, associate with transcriptional machinery, and regulate ER-dependent signalling [62-65], however, treatment with the pure antiestrogens, fulvestrant and ICI 164,384, disrupts nuclear localization of the ER and shuttling of the ER between the cytoplasm and the nucleus [66]. Furthermore, ER agonists induce an ordered distribution of ER within the nucleus, whereas ER antagonists result in a nuclear distribution that is more random than that of an agonist [64,67]. We indeed found that the *Cyclopia* extracts were more efficient at inducing nuclear localization of ERβ (Chapter 4), but not as effective as E_2 at inducing nuclear localization of ERα, and induced a less ordered nuclear distribution of ERα than E_2, while, like E_2, inducing a more ordered nuclear distribution of ERβ. These findings could thus indeed provide an additional explanation for the SERSM mechanism of action of the *Cyclopia* extracts and specifically, supports the ERα antagonist and ERβ agonist behaviour in our transactivation model.

The ER subtypes stimulate the transcription of both common and distinct subsets of E_2 target genes [39,41,44,68-70] and therefore, to further support our SERSM findings, we look towards results from our PCR array to identify genes previously found to be distinctly regulated by either the ERβ or ERα subtype (Chapter 5). Unfortunately, the previous tested arrays [39,41,44,45] differed from the one that we used and thus we could only identify one gene, the cyclin A1 gene (CCNA1), as a distinctly ERβ regulated gene. However, reports differ on how this gene is regulated by E_2 via ERβ with Chang *et al.* [39] reporting up-regulation of CCNA1, whereas Paruthiyil *et al.* [41] reports

down-regulation. In accordance with Chang *et al.* [39], P104 up-regulated CCNA1 expression, while SM6Met down-regulated CCNA1 expression, in accordance with Paruthiyil *et al.* [41]. However, *in vivo* the *Cyclopia* extracts did display ERα antagonism (Chapter 3) by retarding uterine growth [71,72], which supports our ERα antagonism findings.

In conclusion, we have found that the *Cyclopia* extracts display SERSM behaviour. The predominantly ERβ agonism and ERα antagonism of the extracts was illustrated in the transactivation, and to a lesser extent in the transrepression models, was reflected in the differential regulation of ER subtype protein levels as well as nuclear localization and distribution, and was reiterated in the regulation of ERβ regulated genes and the retardation of uterine growth.

The subtype specific effect of the *Cyclopia* extracts on ER protein levels (Chapter 4) also highlights the possibility of SERD development from the *Cyclopia* extracts. All of the *Cyclopia* extracts decreased the ERα:ERβ ratio when compared to that of solvent treated cells, although the values for SM6Met did not reach significance. Treatment with E_2 had a similar effect on the ERα:ERβ ratio, however, this end-point was obtained by robustly down-regulating ERα protein levels while having no significant effect on ERβ levels, while the *Cyclopia* extracts, not only down-regulated ERα, but also increased ERβ protein levels. Co-treatment with E_2 and the *Cyclopia* extracts did not result in additional significant changes in the ERα:ERβ ratio, although it does appear as if co-treatment with SM6Met does further decrease the ERα:ERβ ratio. The effect of E_2 and the *Cyclopia* extracts on ERα protein levels was, however, not reflected in mRNA levels (Chapter 5), a finding supported by other studies [25,73], however, ERβ mRNA expression levels after treatment with P104 and SM6Met, but not cup-of-tea, do reflect effects on ERβ protein levels. These results obtained with the *Cyclopia* extracts reflect those obtained with fulvestrant, a SERD and full ER antagonist, which has been shown to promote the degradation of ERα while stabilizing ERβ protein levels [25,74]. In addition, higher ERα levels are associated with malignant tumours, while higher ERβ levels are

associated with benign tumours [42,75] and lower ERα:ERβ ratios can reduce ERα mediated cell proliferation [76-78], which suggests that the *Cyclopia* extracts may have an inhibiting effect on breast cancer cell proliferation.

Cell proliferation is a hallmark of cancer and in MCF-7BUS cells cell proliferation constitutes an integrated model where not only are the ER subtypes co-expressed, but both transactivation and transrepression of endogenous genes contribute towards the final phenotype, whether it is proliferative or anti-proliferative [39,79-81]. All of the *Cyclopia* extracts, like E_2, induced the proliferation of MCF-7BUS cells, but with lower potencies and efficacies than E_2 (Chapter 3). These results are supported by results obtained with the PCR array of MCF-7BUS cells (Chapter 5) where evaluation of the functional grouping of genes that influence cell growth showed that E_2 regulated 79% of the E_2 regulated genes towards the promotion of cell growth, whereas the *Cyclopia* extracts regulated only 57-59% of the extract regulated genes towards the promotion of cell growth. Furthermore, Ki-67, a proliferation marker [82], is up-regulated to a lesser extent by the *Cyclopia* extracts than by E_2. In addition, we found that, although in the absence of E_2 the *Cyclopia* extracts induced MCF-7BUS cell proliferation, in the presence of E_2, they antagonised E_2-induced cell proliferation (Chapter 3). A possible explanation for this observed antagonism may be found in the SERSM behaviour of the *Cyclopia* extracts. It is known that ERα is associated with the promotion of cell proliferation whereas ERβ inhibits ERα-dependent cell proliferation [37-45]. Thus we are presented with some possible explanations for the observed antagonism. Firstly, the *Cyclopia* extracts may be behaving as ERα antagonists, antagonising E_2-induced cell proliferation. Secondly, the *Cyclopia* extracts may be behaving as ERβ agonists, ameliorating the proliferative effect of ERα. Lastly, the *Cyclopia* extracts may be inhibiting the proliferative effect of ERα by degrading ERα protein levels and enhancing the ameliorative effect of ERβ by stabilizing ERβ

protein levels. Furthermore, the possibility that all of these effects contribute to the inhibition of MCF-7BUS cell proliferation cannot be excluded.

The MTT assay used to evaluate cell proliferation relies on the metabolic activity of proliferating cells and may produce false positive signals due to a test substance inducing metabolic changes in the cell and not due to an increase in proliferation [83]. Cell cycle analysis, in contrast, is a more accurate test system that, in addition, provides new information concerning the phase of cell cycle arrest [83]. For the benefit of the reader, cell proliferation is dependent on the progression of cells through the cell cycle, which is coordinated at certain points, allowing for the necessary regulation of cell growth [84,85]. The *Cyclopia* extracts, like E_2, induced cell proliferation by promoting the movement of cells from the G0/G1 phase to the S phase, but decreased the rate of proliferation by, unlike E_2, either arresting cells in the S phase of the cell cycle or by decreasing the rate whereby cells move from the S phase to the G2/M phase. These findings are supported by our PCR array results (Chapter 5). Specifically, concerning the movement of cells from the G0/G1 to the S phase, all of the *Cyclopia* extracts up-regulated cyclin E1 mRNA levels, a regulator of G0/G1 to S phase transition [86], although not to the same extent as E_2. In addition, the *Cyclopia* extracts, unlike E_2, down-regulated CDKN2A mRNA levels, a negative regulator of G1/S transition cyclins as well as cell proliferation [87], Furthermore, in Chapter 5, we postulated that, concerning the accumulation of cells in the S phase of the cell cycle, the extracts of *Cyclopia* may be disrupting the cell cycle by arresting cells in the S phase by either down-regulating CDK2 (CDK2 inhibition prevents the initiation of DNA synthesis [88]) or by up-regulating ATM mRNA levels (inhibitor of CDK2 [88,89]). Furthermore, upon co-treatment of the cells with the *Cyclopia* extracts and E_2, we found no significant deviation from E_2-induced phase distribution with P104 and cup-of-tea except for a slight increase in the percentage apoptotic cells. However, SM6Met, like the SERM, tamoxifen [90], induced a significant G0/G1 cell cycle phase arrest. We may therefore postulate that in the

presence of E_2, P104 and cup-of-tea attenuates E_2-induced MCF-7BUS cell proliferation by inducing cell death via apoptosis, whereas SM6Met disrupts the cell cycle by arresting cells in the G0/G1 phase of the cell cycle.

Inflammation is considered as an enabling characteristic in cancer development and progression as it contributes to the acquisition of hallmark capabilities in cancer development [81] and, in addition, NFκB, a pro-inflammatory transcription factor, is involved in the development of breast cancer [91-93]. In MCF-7BUS cells, all of the *Cyclopia* extracts displayed anti-inflammatory behaviour in transrepressing an NFκB-containing promoter reporter construct derived from the NFκB element in the promoter of the IL6 gene [94] (Chapter 3). In addition, all of the extracts also down-regulated IL-6 mRNA levels (Chapter 5). Together these results showing that the *Cyclopia* extracts possess anti-inflammatory properties in an environment where both ER subtypes are co-expressed, as in approximately 60% of breast cancer tumours [95-97], may be seen as a positive attribute, as inflammation is considered to be an enabling characteristic for cancer development [81]. In addition, with regards to the postmenopausal surge in inflammatory disorders, the fact that the *Cyclopia* extracts displayed anti-inflammatory behaviour may also be considered a positive attribute, while, in addition, this attribute may also decrease the occurrence of osteoporosis.

Tumour invasion, a hallmark of cancer progression [81], is a process whereby cancer cells from a primary tumour invade surrounding tissues and migrate to distant sites and thereby spread cancer through the body [98]. Furthermore, angiogenesis is employed by tumours to provide nutrients and oxygen and remove carbon dioxide and metabolic waste [81,99]. We found that although one of the *Cyclopia* extracts, P104, regulated the majority of the regulated genes towards angiogenesis (67%), the other two extracts, cup-of-tea and SM6Met, only regulated 22% and 8%, respectively, of the regulated genes towards angiogenesis (Chapter 5). Furthermore, neither E_2, nor the *Cyclopia* extracts, regulated the expression of invasion associated genes in a way that allowed us to

conclusively predict the outcome of *Cyclopia* extract treatment on MCF-7BUS cell invasion (Chapter 5). This is supported by our findings that neither E_2, nor the *Cyclopia* extracts, affected the number of invasive MCF-7BUS cells in a cell invasion assay (Chapter 5). However, we did find that in the presence of E_2, similarly to ICI 182,780 (an ER antagonist) treated cells, the number of invasive cells were increased by the *Cyclopia* extracts. As Goto *et al.* [100] proposed that ERα protects against breast cancer cell invasion and reported an increase in the number of invasive cells by treatment with ICI 182,780, which he ascribed to ERα antagonism, we may speculate that the protective effect of ERα is abrogated by the *Cyclopia* extracts acting as ERα antagonists or by down-regulating ERα. This finding may thus further support our findings that the *Cyclopia* extracts behaved as ERα antagonists (Chapter 3). However, although the observed ERα antagonism may be beneficial for the treatment and prevention of breast cancer during initiation and promotion, it may have a negative effect on breast cancer progression and, therefore, it would be of benefit to evaluate the effect of the *Cyclopia* extracts on breast cancer during different stages of breast cancer development.

For the preparation of an orally administered nutraceutical, such as proposed for the phytoestrogenic *Cyclopia* extracts, a pre-requisite would be intestinal absorption, biological activity *in vivo*, and little to no toxicity at biologically active concentrations. Using the immature rat uterotrophic assay, we for the first time showed that the phytoestrogenic extracts of *C. subternata* are not toxic at the administered doses, are absorbed when administered orally and elicit a biological effect *in vivo* (Chapter 3). Specifically, *Cyclopia* extracts, like E_2, had no effect on liver weights (a surrogate measurement for toxicity), however, in contrast to E_2, the *Cyclopia* extracts delayed vaginal opening, did not induce uterine growth and antagonized E_2-induced uterine growth. These results suggest that the *Cyclopia* extracts are absorbed, are not toxic, and display biological

activity *in vivo* by retarding uterine growth, which supports our previous ERα antagonism findings [71,72].

To conclude, our study expands our current knowledge concerning the behaviour and the molecular mechanism of the phytoestrogenic extracts of *Cyclopia* and identifies the ER subtypes as important molecular targets involved in the development and progression of breast cancer. Specifically, highlights of this study include that these extracts antagonize E_2-induced cell proliferation both *in vitro* and *in vivo*, behave as ERα antagonists and ERβ agonists, and act as SERDs by down-regulating ERα protein levels while stabilizing ERβ protein levels. Therefore, with respect to the known roles of the ER subtypes in breast cancer [37-45], the *Cyclopia* extracts, by behaving as SERSMs, may be beneficial for the prevention or treatment of breast cancer.

Cyclopia extracts have previously been shown to be anti-mutagenic by inhibiting tumour promotion in mouse skin [101], inhibiting aflatoxin B_1- [102] and fumonisin B_1-induced cancer in rat livers [103], and inhibiting esophageal cancer development in rats [104]. However, none of these studies investigated the molecular targets involved in cancer initiation, promotion, and progression and thus the current study is the first to do so. Therefore, although we do believe that the *Cyclopia* extracts show potential to be developed as SERSMs for the treatment or prevention of breast cancer or as a nutraceutical for the alleviation of menopausal symptoms, future work is needed to further establish their molecular mechanism of action. This includes, but is not limited to, directly comparing the *Cyclopia* extracts with known SERMs and SERDs, such as tamoxifen and fulvestrant, investigating the effect of *Cyclopia* extracts on ER homo- or heterodimerization using the BRET assay [95], investigating whether the *Cyclopia* extracts regulate ERα and ERβ specific genes, and evaluating the modulation of cancer development and progression by the *Cyclopia* extracts in a rat breast cancer model such as the MNU-induced mammary gland carcinogenesis model [105]. In addition, further work is needed to identify the polyphenol(s) present in these *Cyclopia* extracts responsible

for eliciting the observed effects, while the possibility that a combination of polyphenols present in *Cyclopia*, rather than an individual polyphenol, may be causing the observed ERα agonism and ERβ antagonism cannot be excluded.

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Addendum A

Phytoestrogenic Potential of *Cyclopia* Extracts and Polyphenols.

Ann Louw, Elizabeth Joubert, Koch Visser

Planta Medica 2013; 79: 580–590

With regard to Addendum A, pp. 227-237, the nature and scope of my contribution were as follows:

Nature of contribution	**Extent of contribution (%)**
Conceived and designed paper Wrote paper: • Tables 2,3,4 & 5 • Section on phytoestrogenic *Cyclopia* extracts under heading Phytoestrogenic potential of *Cyclopia* polyphenols and extracts (812/4345 words) Researched subject Interpreted findings Revised article critically for important intellectual content	• 80% of all Tables • 19% of word count

The following co-authors have contributed to Addendum A, pp. 227-237:

Name	**e-mail address**	**Nature of contribution**	**Extent of contribution (%)**
Ann Louw	al@sun.ac.za	Conceived and designed paper Wrote paper: • Fig. 3 • 3154/4345 words Researched subject Interpreted findings Revised article critically for	• 33% of all Figs • 72% of word count
Elizabeth Joubert	joubertl@arc.agric.za	Conceived and designed paper Wrote paper: • Section on Phenolic Composition of *Cyclopia* (379/4345 words) • Table 1 • Figs. 1 & 2 Interpreted findings Revised article critically for important intellectual content	• 9% of word count • 20% of all Tables • 67% of all Figs

Signature of candidate: J.A.K. Visser

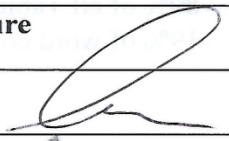
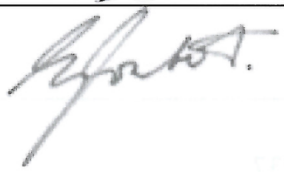
Date: 06-11-13

Declaration by co-authors:

The undersigned hereby confirm that

1. the declaration above accurately reflects the nature and extent of the contributions of the candidate and the co-authors to Addendum A, pp. 227-237,

2. no other authors contributed to Addendum A, pp. 227-237, besides those specified above, and
3. potential conflicts of interest have been revealed to all interested parties and that the necessary arrangements have been made to use the material in Addendum A, pp. 227-237, of this dissertation.

Signature	**Institutional affiliation**	**Date**
	Stellenbosch University	06-11-13
	University of Stellenbosch, Stellenbosch & Agricultural Research Council of South Africa	06-11-13

Phytoestrogenic Potential of *Cyclopia* Extracts and Polyphenols

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Key words
- Fabaceae
- *Cyclopia*
- phytoestrogen
- ER binding
- ERE promoter reporter assay
- E-screen
- uterotrophic assay

Abstract

Cyclopia Vent. species, commonly known as honeybush, are endemic to Southern Africa. The plant is traditionally used as an herbal tea but several health benefits have recently been recorded. This minireview presents an overview of polyphenols found in *Cyclopia* and focusses on the phytoestrogenic potential of selected polyphenols and of extracts prepared from the plant.

Introduction

Cyclopia species (family Fabaceae; tribe Podalyrieae) are part of the fynbos biome and endemic to the coastal and mountainous regions of the Western and Eastern Cape Provinces of South Africa. The plant may grow up to heights of 3 m in the wild and is distinguished by trifoliate leaves and sweet smelling deep yellow flowers with an indented calyx [1] (**Fig. 1**). Although more than twenty species of *Cyclopia* have been described [2], the commercially important species include *C. genistoides, C. sessiliflora, C. intermedia*, and *C. subternata*. Fermented (oxidised) *Cyclopia* is traditionally used as an herbal tea, called honeybush tea, which is acclaimed for its distinct sweet aroma and fragrant flavour. Recently, unfermented honeybush has also been added to the market. *Cyclopia* is one of the few South African plants to have made the transition from regional use to commercial product [3], and in 2011 a total of 174 tons of *Cyclopia* was exported, mostly to Germany (37%), the Netherlands (29%), USA (14%), and UK (12%) (data supplied by Soekie Snyman, SA Rooibos Council, 2012).

Cyclopia has traditionally also been used for medicinal purposes, including as a restorative, as an expectorant, and to promote appetite [4]. Research into the phenolic composition of *Cyclopia* spp. [5–7] has been crucial in identifying value-adding opportunities in the arena of health promoting attributes. Foremost amongst these have been the demonstration of antioxidant properties [8,9], inhibition of tumour development [10,11], and antidiabetic potential [12,13]. Furthermore, scrutiny of phenolic composition coupled to anecdotal claims of *Cyclopia* as of use in stimulating milk production [14] and alleviating menopausal symptoms has led to recent research on the phytoestrogenic potential of *Cyclopia*. This minireview will focus on the polyphenol content of *Cyclopia* and the phytoestrogenic potential of selected polyphenols identified in this genus and extracts from the shoots and leaves of the plant.

Phenolic Composition of *Cyclopia*

The phenolic composition of a number of commercially important *Cyclopia* species has been investigated due to the relevance of these constituents for bioactivity of their herbal teas and extracts. In-depth studies, making use of NMR to unequivocally elucidate chemical structures, deal only with *C. intermedia* and *C. subternata* [5–7, 15]. Generally, *Cyclopia* species are characterised by the presence of the xanthone, mangiferin, with the co-occurrence of its 4-*C*-glucoside regioisomer, isomangiferin, and the flavanone, hesperidin, an *O*-rutinoside of hesperetin, in relatively large quantities [16]. Other classes of compounds identified in *C. intermedia* are flavonols, flavones, isoflavones, and coumestans, as well as some C6-C1 and C6-C2 secondary metabolites [5,6]. Apart from luteolin, none of the latter compounds has been found in detectable quantities in *C. intermedia* extracts by HPLC analysis. The isoflavone orobol was isolated from *C. subternata* [7]. In an *in vitro* culture, *C. subternata* produces glucosides of the isoflavone aglycones, calycosin, pseudobapti-

received Dec. 14, 2012
revised February 19, 2013
accepted March 14, 2013

Bibliography
DOI http://dx.doi.org/10.1055/s-0032-1328463
Published online April 22, 2013
Planta Med 2013; 79: 580–590

ISSN 0032-0943

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Fig. 1 Shoots of *C. subternata* (left) and *C. genistoides* (right) with distinctive yellow flowers having an indented calyx, characteristic of *Cyclopia* species. (Color figure available online only.)

genin, and formononetin, present in *C. intermedia* [5,15]. Recent investigations demonstrated the presence of benzophenones and dihydrochalcones in *C. subternata* [15,17]. An iriflophenone-di-*O*,*C*-hexoside, an eriodictyol-di-*C*-hexoside, 3-hydroxyphloretin-3,5-di-*C*-hexoside, and vicenin-2 (apigenin-6,8-di-*C*-glucoside) were tentatively identified in *C. subternata*, based on UV-Vis, LC-MS, and LC-MS/MS characteristics of the compounds [17]. **Fig. 2** depicts phenolic compounds present in *C. subternata*.

The abundance of *C*-glycosides, both in terms of content and number of compounds (**Fig. 1**, **Table 1**), has implications concerning stability during processing and *in vivo*. The *C*-*C* bond is very stable and resistant to acid and intestinal enzymes able to hydrolyse *O*-glycosides, but evidence of *C*-*C* bond-cleaving reactions by human intestinal bacteria is growing [18–20].

Relatively high levels of certain phenolic compounds are present in the leaves of *C. subternata* (**Table 1**). These values could vary substantially as recently demonstrated by De Beer et al. [17] for seedling plants. Several of the compounds, including mangiferin, isomangiferin, iriflophenone-3-*C*-glucoside, scolymoside, the 7-*O*-rutinoside of luteolin, and eriocitrin, the 7-*O*-rutinoside of eriodictyol, occur in higher levels in aqueous extracts prepared from the leaves, while hesperidin, the 7-*O*-rutinoside of hesperetin, and the dihydrochalcone *C*-glycosides are predominant in the stems. Although natural variation is a contributing factor, trace or undetectable quantities of luteolin by HPLC-DAD in aqueous extracts, whilst present in the methanol extract (**Table 1**), are attributed to poor solubility of this aglycone in water.

Phytoestrogenic Potential of *Cyclopia* Polyphenols and Extracts

Phytoestrogenic potential may be defined in terms of the mechanism of action of the endogenous hormone 17β-estradiol (E_2) [21]. According to this definition, compounds with phytoestrogenic potential would act through at least one of the main isoforms of the estrogen receptor (ER), namely ERα or ERβ [22], and act as agonists, antagonists, or selective ER modulators (SERMS) via ER signalling pathways [21] (**Fig. 3**). Phytoestrogens are, however, also considered to be endocrine disruptors and as such the definition used by regulatory bodies in both the USA and Europe could be useful [23,24]. The European Commission State of the Art Assessment of Endocrine Disruptors, for example, defines estrogenicity in terms of "binding to the estrogen receptor(s) (ER), ER activation, cell proliferation in ER-competent cells and physiological responses (proliferation of uterine tissue in rodents, induction of vitellogenin in fish)" [24].

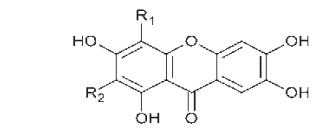
Although several assays have been suggested to evaluate estrogenic activity [25], for the purposes of this review we will evaluate the phytoestrogenic potential of both the polyphenols shown to be present in *Cyclopia* and extracts prepared from *Cyclopia* in terms of their *in vitro* ability to either bind to ERα or ERβ, to induce or prevent activation of ER-responsive promoters, or to cause cell proliferation in ER-responsive cells (e.g., E-screen in MCF-7 cells, a breast cancer cell line) or in terms of their *in vivo* responses in known estrogenic tissues such as the uterus (**Fig. 3**, **Tables 2**, **3**, and **4**). In addition, where it was not apparent that the ER was involved, we used evidence of loss of activity via ICI 182,782, an ER antagonist, as confirmation of ER involvement.

Although *in vivo* studies have been considered the "gold standard" for the evaluation of estrogenicity, many authors have not conducted such studies, and thus we have to rely on *in vitro* results. In terms of *in vitro* results, it is important to establish that

Table 1 Phenolic composition of leaves and extracts (g · 100 g^{-1} dry basis) of unfermented *Cyclopia subternata*.

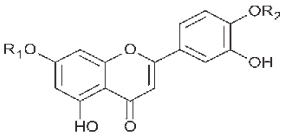
Compound	Leaves [92] (n = 6)	Aqueous extract [16] (n = 6)	Aqueous extract [17] (n = 64)	Methanol extract [44] (n = 1)
Mangiferin	1.22 ± 0.35	2.73 ± 1.65	0.93 ± 0.42	1.91
Isomangiferin	0.38 ± 0.05	0.86 ± 0.28	0.47 ± 0.12	0.77
Hesperidin		0.62 ± 0.17	0.64 ± 0.36	2.21
Eriocitrin	0.23 ± 0.06	0.32 ± 0.07	0.55 ± 0.15	1.25
Eriodictyol glucoside[a]		0.35 ± 0.07[b]		
Iriflophenone-3-*C*-β-glucoside	0.25 ± 0.06	0.82 ± 0.44[c]	0.47 ± 0.29	
3-Hydroxyphloretin-3,5-di-*C*-hexoside[a]			0.54 ± 0.13	
Phloretin-3,5-di-*C*-glucoside	0.41 ± 0.01	0.86 ± 0.20[d]	1.05 ± 0.34	1.22[f]
Scolymoside	0.48 ± 0.32	0.68 ± 0.62[e]	0.49 ± 0.24	2.04[g]
Luteolin				0.09

[a] Position and/or identity of glycosyl moiety not certain; previous designation, [b] compound **9**, [c] compound **8**, [d] compound **12**, [e] compound **11**, [f] unknown **2**, [g] unknown **1**



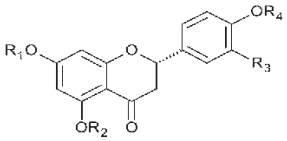
Xanthone

Mangiferin: R_2 = *C*-glucosyl; R_1 = H
Isomangiferin: R_2 = H; R_1 = *C*-glucosyl



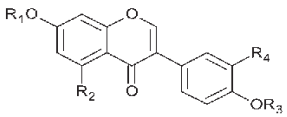
Flavone

Luteolin: R_1, R_2, = H
Diosmetin: R_1 = H; R_2 = CH_3
Scolymoside: R_1 = rutinosyl; R_2 = H



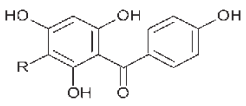
Flavanone

Naringenin: R_1, R_2, R_3, R_4 = H
Eriodictyol: R_1, R_2, R_4 = H; R_3 = OH
Hesperetin: R_1, R_2 = H; R_3 = OH; R_4 = CH_3
Narirutin: R_1 = rutinosyl; R_2, R_3, R_4 = H
Prunin: R_1 = glucosyl; R_2, R_3, R_4 = H
*Eriodictyol-*O*-glucoside: R_1 or R_2 = glucosyl or H; R_3 = OH, R_4 = H
Hesperidin: R_1 = rutinosyl; R_2 = H; R_3 = OH; R_4 = CH_3
Eriocitrin: R_1 = rutinosyl; R_2, R_4 = H; R_3 = OH



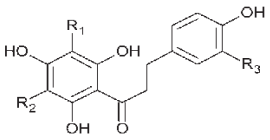
Isoflavone

Orobol: R_1, R_3 = H; R_2, R_1 = OH
Formononetin: R_1, R_2, R_4 = H; R_3 = CH_3
Calycosin: R_1, R_2 = H; R_3 = CH_3; R_4 = OH
Ononin: R_1, = glucosyl; R_2, R_4 = H; R_3 = CH_3
Calycosin-7-*O*-glucoside: R_1 = glucosyl; R_2 = H; R_3 = CH_3; R_4 = OH



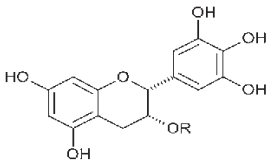
Benzophenone

Iriflophenone-3-*C*-β-glucoside: R = glucosyl



Dihydrochalcone

Phloretin-3',5'-di-*C*-β-glucoside: R_1, R_2 = *C*-glucosyl; R_3 = H
*3-Hydroxyphloretin-3',5'-di-*C*-hexoside: R_1, R_2 = *C*-hexoside; R_3 = OH



Flavanol

Epigallocatechin gallate: R = gallate

Fig. 2 Structures of major phenolic compounds of *C. subternata* and minor compounds with estrogenic activity present in the leaves and stems of some *Cyclopia* spp. (* indicates that the position or identity of the glycosyl moiety is not certain; bold text indicates the class of compound).

a hierarchy in terms of sensitivity has been established, with the E-screen generally considered the most sensitive assay [26–28]. Furthermore, although binding to the ER may be considered a prerequisite for estrogenic activity and is certainly the most characteristic mode of action of phytoestrogens [29], receptor binding assays cannot distinguish agonists from antagonists or SERMs [26]. Assays relying on the activation of ER-responsive promoters (both of artificial ERE-containing promoter reporters and endogenous ERE-containing estrogen responsive genes) and the E-screen are more appropriate assays to distinguish agonists from antagonists and SERMs [26]. Furthermore, to distinguish activation of ERα from activation via ERβ, cell lines expressing these receptors separately have to be utilised. MCF-7 cells, used in the E-screen, contain both ERα and ERβ and thus lack the ability to discriminate between the roles of the ER isoforms [25]. In addition, the uterotrophic assay is primarily an assay to verify ERα-mediat-

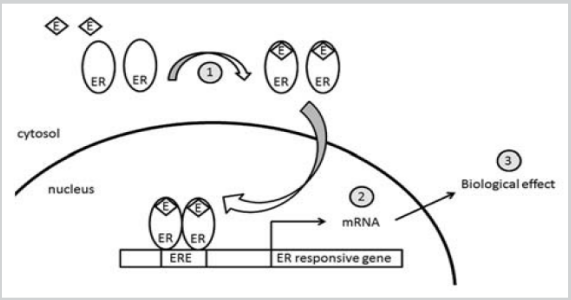


Fig. 3 Steps in ER signalling used to evaluate estrogenicity. E = estrogenic compound, ER = estrogen receptor, ERE = estrogen response element. (**1**) Binding of an estrogenic ligand to the ER may be evaluated by ligand-binding assays, (**2**) binding of ligand-activated ER to an ERE in the promoter of an estrogen responsive gene may be evaluated by promoter-reporter studies using an ERE-containing promoter reporter or by measuring mRNA levels of select ER-responsive genes, and (**3**) downstream biological effects such as cell proliferation or hypertrophy of the uterus may be measured using the E-screen or uterotrophic assay, respectively.

Table 2 Known [5–7, 15] *Cyclopia* polyphenols that have not been tested for estrogenic potential.

Class of compound	Specific compound(s)
Xanthone	isomangiferin
Flavanone	eriodictyol-5-*O*-glucoside, eriodictyol-7-*O*-glucoside, naringenin-5-*O*-glucoside, isosakuranetin
Flavone	5-deoxyluteolin, scolymoside, isorhoifolin, vicenin-2
Flavonol	kaempferol-5-*O*-glucoside, kaempferol-6-*C*-glucoside, kaempferol-8-*C*-glucoside
Methylinedioxyflavanol derivative	3′4′-methylinedioxyflavanol apiosyl-glucoside
Isoflavone	formononetin apiosyl-glucoside, afrormosin, rothindin, wistin
Methylinedioxyisoflavone derivative	pseudobaptigenin, fujikinetin
Coumestan	flemichapparin, sophoracoumestan B
Benzophenone	iriflophenone-3-*C*-*β*-glucoside
Dihydrochalcone	phloretin-3′,5′-di-*C*-*β*-glucoside
Benzaldehyde derivative	benzaldehyde apiosyl-glucoside
Phenylethanoid derivative	tyrosol,3-methoxy-tyrosol, 4-glucosyltyrosol, phenylethanol apiosyl-glucoside

ed *in vivo* effects, and no appropriate *in vivo* assay for ER*β* has been established [25].

Initially, we wanted to standardise our comparison of the estrogenic potential of polyphenols in *Cyclopia* using the relative binding affinity (RBA) and relative induction index (RII) where binding and activation are expressed relative to the values for E_2 (calculated as follows: $100 \times IC_{50}$ or EC_{50} (E_2)/IC_{50} or EC_{50} (test compound), however, we found that few papers provide quantitative data. Thus most of our comparisons of estrogenic activity of the polyphenols present in *Cyclopia* (**Table 3**) rest on qualitative and not quantitative data.

Most of the polyphenols present in *Cyclopia* have, to our knowledge, not been tested for estrogenicity (**Table 2**). For example, the dihydrochalcone phloretin-3′,5′-di-*C*-*β*-glucoside, the flavone scolymoside, and the benzophenone iriflophenone-3-*C*-*β*-glucoside, all present in relatively high concentrations in *C. subternata* (**Table 1**), have not been tested (**Table 2**).

Table 3 summarises data for compounds that have been tested for estrogenicity in different assay systems. Mangiferin, the major xanthone in *Cyclopia* species (**Table 1**), has been shown to have no estrogenic activity both via ER binding assays and ERE-promoter reporter assays (**Table 3**). Although isomangiferin has not been tested (**Table 2**), it is unlikely to have estrogenic activity as it is a regioisomer of mangiferin (**Fig. 2**). The phenolic acid p-coumaric acid and the coumestan medicagol have both been tested but found not to be estrogenic (**Table 3**).

Of the flavanones present in *Cyclopia*, most have been tested for estrogenicity. Prunin (naringenin-7-*O*-glucoside), one of the rarer flavanones, is estrogenic, while of the glycosylated flavanones present in relatively high concentrations in *Cyclopia* (**Table 1**), like eriocitrin and hesperidin, only eriocitrin is estrogenic (**Table 3**). Eriodictyol and naringenin, as well as their rutinosyl derivatives, eriocitrin and narirutin bind to ER, although therutinosyl derivatives bind with a lower affinity than their corresponding aglycones. Specifically, in a competitive binding assay, eriodictyol and naringenin displaced 44% and 70% of 1 nM tritiated E_2 from ER*β*, respectively, while their corresponding rutinosyl derivatives displaced 28% and 28%, respectively [30]. Naringenin is interesting as it has been shown to be estrogenic *in vitro* using the usual array of screening assays, namely ER-binding, activation of ERE-responsive promoters both in promoter reporter studies and with endogenous genes, yet *in vivo*, using the immature uterotrophic assay, it does not display estrogenicity (**Table 3**). This may suggest that naringenin is not absorbed or is inactivated, either during hepatic metabolism or by gut bacteria, and highlights the importance of validating these parameters [31]. On the other hand, it may also suggest that naringenin does not transactivate via ER*α*, the ER responsible for uterotrophic action, but rather via ER*β*, as borne out by some [32], but not by other [33–35] promoter reporter studies. Hesperetin and its rutinosyl derivative, hesperidin, do not bind ER, although hesperetin, but not hesperidin, does transactivate an ERE-containing promoter reporter, which can probably be ascribed to the lower activity of glycosalyted derivatives relative to their aglycones. Furthermore, hesperetin activates estrogen responsive genes and causes cell proliferation in the E-screen via an ER-mediated mechanism as ICI 182,782 antagonises the response. This suggests that the ER-binding assay may not be sensitive enough to evaluate weak estrogenicity, which is further borne out by the fact that in three studies where naringenin and hesperetin were directly compared, hesperetin was a weaker agonist [33, 34, 36]. Specifically, Breinholt and Larsen [36] report EC_{50} values of 89.6 µM and 0.3 µM, while Promberger et al. [34] report 2% and 80% efficacy for hesperetin and naringenin, respectively, in ERE-containing promoter reporter studies. Liu et al. [33] also clearly show that hesperetin is weaker than naringenin at causing both cell proliferation in the E-screen and activation in promoter reporter studies. The lower activity of hesperetin relative to naringenin may be ascribed to the methyl functional group found on the B-ring of hesperetin (**Fig. 2**). The flavanol (−)-epigallocatechin gallate, however, was found to be estrogenic by binding to ER and via the GAL4 promoter assay (a very artificial system in which the ER is fused to a GAL4 element), but not via the ERE-containing promoter reporter assay (**Table 3**). This suggests that, contrary to what we have suggested for hesperetin, namely that ER binding may not be sensitive enough to test for weak estrogenic activ-

Table 3 Phytoestrogenic potential of polyphenols found [5–7, 15] in *Cyclopia*.

Polyphenol	Estrogenic effect	Test for estrogenic effect		Reference
		Test system	Test model	
Xanthones				
Mangiferin	No	ER binding assay	COS-1 cells + hERα or hERβ	[30, 32]
			Fluorescence ERα competitor assay kit	[45]
		ERE promoter reporter assay	COS-1 cells + hERα or hERβ	[32]
Flavanones				
Hesperetin	No	ER binding assay	COS-1 cells + hERα or hERβ	[30]
			MCF-7 cells	[93, 94]
	Yes	ERE promoter reporter assay	Yeast cells + hERα	[34]
			Yeast cells + hER	[36]
			U2OS cells + hERα or hERβ	[33]
		Estrogen responsive genes	PC12 cells ± ICI[a]	[95]
		Cell proliferation assay	MCF-7 cells ± ICI	[33]
Hesperidin	No	ER binding assay	COS-1 cells + hERα or hERβ	[30]
		ERE promoter reporter assay	MCF-7 cells	[43]
Eriodictyol	Yes	ER binding assay	COS-1 cells + hERα or hERβ	[30]
		ERE promoter reporter assay	Yeast cells + hER	[96]
Eriocitrin	Yes	ER binding assay	COS-1 cells + hERα or hERβ	[30]
Naringenin	Yes	ER binding assay	COS-1 cells + hERα or hERβ	[30, 32]
			Nonisotopic ERβ-based assay	[37]
		ERE promoter reporter assay	COS-1 cells + hERα or hERβ	[32]
			MCF-7 cells	[43, 97]
			U2OS cells + hERα or hERβ	[33]
			Yeast cells + hERα; hER; ERα or ERβ	[34, 35, 95]
		Estrogen responsive genes	BT-474 cells	[98]
		Cell proliferation assay	MCF-7 cells ± ICI	[32, 33]
	No	Uterotrophic assay	Immature rats; mice	[34, 84]
Narirutin	Yes	ER binding assay	COS-1 cells + hERα or hERβ	[30]
Prunin	Yes	ERE promoter reporter assay	MCF-7 cells	[43]
Flavones				
Luteolin	Yes	ER binding assay	COS-1 cells + hERα or hERβ	[30, 32]
			Nonisotopic ERβ-based assay	[37]
			MCF-7 cells	[46]
		ERE promoter reporter assay	MCF-7 cells	[43, 46]
			COS-1 cells + hERα or hERβ	[32]
		Estrogen responsive genes	BT-474 cells	[98]
		Cell proliferation assay	MCF-7 cells ± ICI	[32]
Diosmetin	Yes	ERE promoter reporter assay	Yeast cells + hERα	[34]
Isoflavones				
Formononetin	Yes	ER binding assay	hERα or hERβ	[38]
			ERα or ERβ	[99]
			COS-1 cells + hERα or hERβ	[30, 32]
			Nonisotopic ERβ-based assay	[37]
	No		Rabbit uterine estrogen receptor	[100]
	Yes	ERE promoter reporter assay	COS-1 cells + hERα or hERβ	[32]
			MCF-7 cells ± ICI	[43, 101]
			Yeast cells + hERα; hERα or hERβ	[34, 40, 102]
		Cell proliferation assay	MCF-7 cells ± ICI	[32, 101]
		Uterotrophic assay	Ovariectomised mice	[41]
Calycosin	Yes	ER binding assay	Erα and Erβ competitor assay kit	[38]
		ERE promoterreporterassay	MCF-7 cells	[42]
		Uterotrophic assay	Ovariectomised mice	[41]
Calycosin-7-*O*-glucoside	Yes	ERE promoter reporter assay	MCF-7 cells	[43]
Orobol	Yes	ER binding assay	ERα and ERβ competitor assay kit	[103]
			ERα or ERβ	[104]
		ERE promoter reporter assay	Yeast cells + hERα	[105]
			U2OS cells + hERα	[105]
Ononin (formononetin-7-*O*-glucoside)	Yes	ERE promoter reporter assay	MCF-7 cells	[43]

continued

Table 3 *Continued*

Polyphenol	Estrogenic effect	Test for estrogenic effect		Reference
		Test system	Test model	
Flavanols				
(−)-Epigallocatechin gallate	Yes	ER binding assay	hERα or hERβ	[94]
			Mouse uterine estrogen receptor	[94]
		Gal4 promoter reporter assay	MCF-7 cells + hERα or mERβ + 17m5-G-Luc	[94]
	No	ERE promoter reporter assay	HeLa cells + hERα or hERβ	[95]
Coumestans				
Medicagol	No	ER binding assay	Rabbit uterine estrogen receptor	[100]
Phenolic carboxylic acid				
p-Coumaric acid	No	Uterotrophic assay	Ovariectomised rats	[106]

[a] ICI 182,782: an estrogen receptor antagonist

ity, some compounds may bind ER but not display estrogenicity in other assays.

Of the flavones present in *Cyclopia* only two, luteolin and diosmetin, have been tested for estrogenicity, and both are estrogenic (**Table 3**). Luteolin is present in a methanol extract from *C. subternata* (**Table 1**) and has been shown to be estrogenic via ER-binding, ERE-containing promoter assays, and estrogen responsive genes, as well as by stimulating cell proliferation in the E-screen. It has, however, not been tested *in vivo*. Work from our laboratory suggests that luteolin binds preferentially to ERβ, with an RBA of 0.52% for ERβ, while for ERα the RBA is 0.0025% [30,32] and that it has a similar affinity for ERβ as naringenin [30,32,37]. In promoter reporter assays, luteolin has a lower potency but higher efficacy via ERβ than naringenin, specifically it has a potency of 3.53×10^{-3} mg/mL (12.3 µM) versus the potency of 1.04×10^{-4} mg/mL (0.0382 µM) of naringenin and a efficacy of 3.69-fold versus a 2.99-fold induction by naringenin. However, unlike naringenin it does transactivate via ERα, with a potency of 1.97×10^{-3} mg/mL (6.88 µM), which is just slightly higher than via ERβ. Yet, in the E-screen, it has a lower potency (2.54×10^{-6} mg/ml or 0.00887 µM) than naringenin (3.27×10^{-8} mg/ml or 0.00012 µM) suggesting that in terms of a biological response in physiologically relevant tissues, it may favour ERβ.

Although the isoflavones shown to be present in *Cyclopia* are not observed in quantifiable amounts (**Fig. 2**, **Table 1**), many of them are estrogenic (**Table 3**). Of these, formononetin and calycosin have been thoroughly tested, both *in vitro* and *in vivo*, and generally show a slight preference for ERβ in ER binding assays [30,32,38,39]. These compounds differ only on the B-ring in that calycosin has a 3′-OH moiety. In promoter reporter studies, the ER isoform preference for formononetin is not so clear [32,40], while both compounds are uterotrophic, with calycosin being more potent than formononetin [41,42], suggesting that both must act via ERα. Here again we observe the phenomenon of the glycoside being less estrogenic than its corresponding aglycone, with calycosin showing greater estrogenic activity via a promoter reporter construct in MCF-7 cells than calycosin-7-*O*-glucoside [43]. Orobol, with OH groups at the 3′ and 4′ positions, and ononin, the 7-*O*-glucoside of formonentin, are also both estrogenic but here their activity appears to be similar to that of calycosin-7-*O*-glucoside and not to be preferentially via ERβ (**Table 3**).

The presence of polyphenols with phytoestrogenic capabilities in the plant material of *Cyclopia* species (**Table 3**) raised the question of whether extracts from the plant material will have phytoestrogenic capabilities. One cannot simply assume that the estrogenicity of the pure compounds will be transferred to extracts of the plant material as varying levels of polyphenols, as well as the presence of various polyphenols with varying levels of estrogenicity, might modulate the effects observed with pure polyphenols. To address this issue, examination of the phytoestrogenicity of crude extracts prepared from the plant material of various commercially cultivated *Cyclopia* species [30,32,44] as well as the HPLC analyses of these extracts to identify the polyphenols present is warranted. We chose two extracts for discussion (**Table 4**), P104 (methanol extract) from *C. genistoides* as it was found to have the highest binding affinity for both the ER subtypes [32], and SM6Met (methanol extract of plant material following extraction with ethyl acetate and ethanol) from *C. subternata* as it had the highest potency when compared to other extracts [44]. P104 bound to both ERα and ERβ, albeit with a lower potency than that of E_2, and had a higher affinity for ERα. This correlates with previous studies that showed a slightly higher displacement of E_2 from ERα than from ERβ by P104 [30]. Despite binding to ERα with a higher affinity, P104 was not able to activate an ERE containing promoter reporter construct through ERα, but was able to do so through ERβ with an efficacy similar to that of E_2, although its potency was much lower. In addition, P104 induced cell proliferation of MCF-7 cells, but it was less potent than E_2. SM6Met has also been shown to bind to the ER by performing whole cell binding assays in MCF-7 cells. Unfortunately, these results cannot distinguish between binding to specific ER isoforms as MCF-7 cells contain both ERα and ERβ. Similar to P104, SM6Met also activated an ERE containing promoter reporter construct and induced cell proliferation in MCF-7 cells and like P104, SM6Met had a lower potency than E_2 in both assays. The extracts were analysed with HPLC, and **Table 4** shows the polyphenols detected. Apart from these, the extracts were also screened for narirutin, eriodictyol, naringenin, hesperetin, and formononetin. Although these polyphenols were not present in quantifiable amounts, one cannot exclude the possibility of their presence and thus the effect they may have on the estrogenicity of the whole extract. The unidentified compounds in the extract of Mfenyana et al. [44] have since been tentatively identified (**Table 4**) as the flavone, scolymoside, and the dihydrochalcone, phloretin 3′,5′-di-*C*-β-glucoside. The presence of unidentified compounds was also previously indicated for P104 [32], but they were not quantified. Comparison of **Tables 3** and **4** may allow the deduction of which of the polyphenols might be causing the phytoestrogenicity of the extracts. Both extracts contain the xanthones mangiferin and isomangiferin, but as they are not phytoestrogenic [30,32,45] (**Tables 2** and **3**), it is unlikely that they are contributing. Hesperidin also does not bind to hERα or hERβ and is un-

Table 4 Phytoestrogenic potential of polyphenols and extracts of unfermented *C. genistoides* and *C. subternata*.

Species		Extract	
		P104 [32]	SM6Met [44]
		C. genistoides	*C. subternata*
ER binding[a] (RBA[b] ± SEM[c])		ERα: 0.1195 ± 0.0567 % ERβ: 0.0004 ± 0.0001 %	0.0802 ± 0.0139 %
ERE promoter reporter assay[d] (RII[e])	Potency ± SEM	ERβ: 1.0490 ± 0.1287 %	0.0102 ± 0.0032 %
	Efficacy ± SEM	ERβ: 103.2 ± 1.1 %	57.6 ± 2.4 %
Cell proliferation assay[f] (RII)	Potency ± SEM	0.0072 ± 0.0069 %	0.0579 ± 0.0325 %
	Efficacy ± SEM	99.1 ± 2.3 %	78.5 ± 6.6 %
Polyphenols (g · 100^{-1} g dry extracts ± SEM)			
▶ Mangiferin		3.935 ± 0.329	1.85
▶ Isomangiferin		4.998 ± 0.097	0.75
▶ Eriocitrin		ND[g]	1.25
▶ Hesperidin		1.503 ± 0.226	1.87
▶ Luteolin		0.097 ± 0.001	0.04
▶ Scolymoside[h]		ND	1.82
▶ Phloretin-3,5-di-*C*-glucoside[i]		ND	1.27

[a] Whole cell bindings were performed in COS-1 cells transfected with hERα or hERβ [32] and in MCF-7 cells that contain both hERα or hERβ [44]. [b] RBA or relative binding affinity is expressed relative to that of E_2 (100 %) and was calculated as follows: 100 × IC_{50} (E_2)/IC_{50} (test compound). [c] Values represent an average of values from different extractions of the same plant material. [d] ERE promoter reporter assays were performed in COS-1 cells transfected with hERα or hERβ [32] or in T47D-KBluc cells that contain both hERα or hERβ [44]. [e] RII or relative induction index is expressed relative to that of E_2 (100 %) and was calculated as follows: 100 × EC_{50} (E_2)/EC_{50} (test compound) for potencies and 100 × fold (test compound)/fold (E_2) for efficacies. [f] Cell proliferation assays were performed in MCF-7 cells. Verhoog et al. performed assays in the presence and absence of ICI 182,782 [32]. [g] Not detected. [h] Previously 'Unknown **1**'. [i] Previously 'Unknown **2**'

able to induce an ERE containing promoter reporter construct [30, 43], however, its aglycone hesperetin, despite showing no binding to ER, does transactivate ERE-containing promoters and causes cell proliferation in the E-screen (**Table 3**). As glycosides are likely to be metabolised to their aglycones *in vivo*, hesperidin should not be discounted for *in vivo* studies, however, for *in vitro* testing, it is unlikely to contribute to the estrogenicity of the extracts. Luteolin has been shown to bind to both ER isoforms [30, 32, 37, 46], to activate an ERE promoter reporter construct through both isoforms [32, 43, 46], and to induce proliferation of a breast cancer cell line (**Table 3**). The amount of luteolin present was, however, shown to be too low to explain the degree of phytoestrogenicity observed for the P104 [32] or SM6Met [44] extract. On the other hand, scolymoside, the 7-*O*-rutinoside of luteolin, may be important *in vivo*. The flavanone eriocitrin was quantified in SM6Met, but not in P104 (**Table 4**). Eriocitrin has been shown to bind to ERβ [30], but no further tests for estrogenicity have been performed (**Table 3**). To our knowledge, scolymoside and phloretin 3′,5′-di-*C*-β-glucoside tentatively identified in SM6Met have not been tested for phytoestrogenicity (**Table 2**). Taken together, no concrete conclusions regarding the polyphenols responsible for the phytoestrogenic effect of extracts of *Cyclopia* can be drawn. Some of the identified polyphenols still need to be tested for phytoestrogenicity, and the desired answer might be found in the results from these studies. We cannot, however, exclude the possibility that the effect seen with the *Cyclopia* extracts is the result of a fine balance between different polyphenols present in varying amounts with varying phytoestrogenic potential (agonistic, antagonistic, or SERM activity via either ERα or ERβ) and that synergism or antagonism could play a role with multiple polyphenols targeting multiple ER isoforms [47].

Blanket Claims for Phytoestrogenic Potential of *Cyclopia*

Caution should be exercised in making blanket claims for the phytoestrogenic potential of all harvestings of *Cyclopia*. Research indicates that variations in the polyphenol composition or content as well as the phytoestrogenic potential of individual harvestings of a specific *Cyclopia* species may differ (**Table 5**). For example, *C. genistoides* dried methanol extracts differed remarkably in their ability to induce cell proliferation in the E-screen assay with three out of the six harvestings displaying such low levels of activity that EC_{50} values could not be determined (**Table 5**). Even amongst the harvestings with higher activity, there was considerable variation with M7 and NP105 extracts displaying 1.4- and 3.3-fold less activity than NP104. In addition, the concentration of luteolin, a polyphenol with proven phytoestrogenic potential (**Table 3**), also varied between harvestings with a 2.6-fold difference between the harvesting with the highest concentration (M9) and that with the lowest concentration (NP104 or NP105) of luteolin (**Table 5**). This variability in polyphenol content is even more pronounced both quantitatively and qualitatively between species of *Cyclopia* with, for example, eriocitrin varying between undetectable in the *C. genistoides* aqueous extract to 0.47 % of the aqueous extract of unfermented *C. subternata* [8].

The lack of standardisation, both in terms of levels of active substances and activity levels, of botanical and dietary supplements plagues the industry. Combined with little to no regulation by national bodies regulating drug use in most countries, this has led to contrary and inconsistent findings relating to health benefits, which has damaged the credibility of the industry [48]. Thus for claims of phytoestrogenic activity in *Cyclopia*, individual harvestings would have to be tested for activity until such time as a marker compound(s) shown to be related to activity can be identified.

Table 5 Variation in phytoestrogenic potential and polyphenol content of *C. genistoides* harvestings.

Farm	Harvesting date	Dried methanol extract	E-screen in MCF-7 cells RII[c]	Luteolin ($g \cdot 100^{-1}$ g dry extracts)
Koksrivier/Overberg[a]	22 January 2002	M7	9.8×10^{-5}	0.13
Reins/Albertina[a]	01 April 2003	M8	ND[d]	0.12
Reins/Albertina[a]	22 April 2004	M9	ND	0.25
Koksrivier/Overberg[b]	15 March 2001	NP104	1.4×10^{-4}	0.097
Koksrivier/Overberg[b]	28 March 2001	NP105	4.3×10^{-5}	0.097
Koksrivier/Overberg[b]	31 March 2003	NP122	ND	0.104

[a] Data from [44]; [b] data from [32]; [c] RII (relative induction index) = EC_{50} E_2/EC_{50} extract; [d] ND = RII could not be determined as activity was too low

Potential Usage of Phytoestrogens

Estrogen plays an important role in the development of the female reproductive tract, secondary sex characteristics, and in reproductive behaviour [49]. However, estrogen also influences the growth of hormone-dependent cancers such as breast cancer [50].

Hormone replacement therapy (HRT), which includes estrogen combined with or without progesterone, is given to alleviate the symptoms of menopause, and advocates of HRT believe that it also confers long-term benefits regarding cardiovascular disease, bone preservation, and general well-being [51,52]. Although the efficacy, superiority, and cost effectiveness of estrogen in the treatment of menopausal symptoms is accepted [53], recent large randomised clinical trials [54,55] and observational studies [56] on HRT have modified the risk/benefit perception. Specifically, increased risk of breast cancer and cardiovascular disease has raised concerns amongst the public [57], and the Endocrine Society statement of 2010 now recommends use of HRT with the lowest effective dose and for the shortest duration possible [58].

The double-edged sword of estrogen has prompted the search for alternatives in the management of menopause, and phytoestrogens have been suggested as a viable alternative, due to their potential to modulate estrogen action [59,60]. In addition, epidemiological studies suggest that Asian populations who consume 20–50 mg soy/day have fewer occurrences of hormone-dependent diseases, including menopausal symptoms, osteoporosis, and breast cancer and that this lower incidence is not due to under reporting or genotypic factors [53,61–63].

Pharmacological validation of claimed health benefits for phytoestrogens has, however, only recently been undertaken and most work has focused on *in vitro* assays to establish biological activity while large, well-designed *in vivo* studies have lagged behind [64]. Molecular aspects of phytoestrogens that have been heralded as positive regarding health benefits include the fact that phytoestrogens generally have orders of magnitude lower potency than estrogen [53,65], display estrogen agonist activities in the presence of low levels of estradiol (post-menopausal) and antagonistic activity in the presence of high levels of estradiol (premenopausal) [48], exhibit partial selectivity for ERβ, the ER isoform believed to attenuate the proliferative effect of ERα [66, 67], and many act like SERMs, making them safer for breast and endometrial tissue [29,48,68]. Furthermore, phytoestrogens have additional diverse beneficial biological effects, such as anti-inflammatory, antioxidant, and anticancer effects [65,69].

Several studies and reviews have evaluated the health potential of phytoestrogens for treating post-menopausal symptoms by maintaining bone density, decreasing cardiovascular disease and hot flashes, and in preventing or treating estrogen-dependent cancers such as breast, prostate, endometrial, and colon cancer [29,48,53,70–73]. Although there is contradictory scientific proof of the effectiveness of phytoestrogens, specifically soy and red clover isoflavones, for the treatment of vasomotor menopausal symptoms, such as hot flushes [29,73,74], for other symptoms, such as osteoporosis and cardiovascular disease, the data to date strongly suggests efficacy. Specifically, phytoestrogens, such as coumestrol, genistein, daidzein and its metabolite equol as well as extracts from soy, black cohosh, and red clover, appear to slow bone loss and improve bone density [29,48], which is positive for osteoporosis, while for cardiovascular disease, phytoestrogens, primarily from soy, are beneficial in decreasing LDL and triglycerides, while increasing HDL [48,53]. In addition, several studies have suggested that phytoestrogen use, mainly flavones and isoflavones from soy, is associated with a reduced risk of breast cancer [67,75–77].

Despite beneficial effects of phytoestrogens being reported, results have, however, not always been favourable or reproducible [73]. For example, although some studies suggest that soy food intake does correlate with reduced risk or recurrence of breast cancer [78,79], other studies have found no such association between isoflavone intake and breast cancer risk [80,81]. The diversity in results may be attributed to, amongst others, the fact that a wide variety and doses of botanicals have been used and the fact that standardisation of formulations are not currently required making comparison between studies difficult [29,48,70]. In addition, an evaluation of effects of phytoestrogenic preparations on health is complicated by the fact that exact formulations and concentrations of active constituents are not always known and studies are often retrospective (relying on recall of diet). Furthermore, the fact that there has never been a study comparable in size to the Million Women's or WHI studies investigating side effects of phytoestrogen use should encourage caution. This is especially relevant as many consumers base their beliefs of both efficacy and safety on source rather than evidence [29]. Despite this caveat, there is no current data suggesting that dietary phytoestrogens promote hormone-dependent cancers in humans, and thus phytoestrogens can probably be used safely on a long-term basis [53,73]. Finally, the fact that phytoestrogens are often not selected for specific attributes, such as acting only via ERβ, may have confounded studies on health effects. Some promising results regarding amelioration of hot flushes with liquiritigenin, an ERβ-selective agonist from a Chinese herbal extract, have, however, resulted in Phase 2 clinical trials to evaluate safety and efficacy for the treatment of menopausal symptoms [82,83].

Conclusions

The increased public and industry interest in phytoestrogens suggests that validated health claims would contribute significantly to adding value to products such as honeybush tea. Certain extracts of *Cyclopia* undoubtedly display estrogenic activity (**Table 4**), and many of the major and minor polyphenols found in *Cyclopia* certainly have been shown to have phytoestrogenic potential (**Table 3**), but whether this translates into firm health recommendations for a "cup-of-tea" of honeybush is debatable. Firstly, harvestings of *Cyclopia* differ significantly in terms of estrogenic activity and polyphenol content (**Table 5**), and secondly, *Cyclopia* extracts have not been tested for estrogenicity *in vivo*. The importance of evaluating the bioavailability as well as the metabolic transformation of active compounds, both by gut microflora and hepatic enzymes, has been stressed [31, 84]. *Cyclopia* extracts have been tested *in vivo* for absorption and metabolism [85, 86]; however, the focus was on mangiferin and hesperidin, both compounds without estrogenic activity (**Table 3**). The aglycone of hesperidin, hesperetin, which does display weak estrogenic activity, was, however, one of the metabolites detected in urine [85]. This suggests that glycosylated polyphenols, of which several constitute the major polyphenols in *Cyclopia* extracts (**Table 1**), would probably be transformed to the corresponding aglycone with higher phytoestrogenic activity. Finally, the concept of either synergistic or even antagonistic formulations consisting of intelligent mixtures of natural products to treat disease is gaining ground [47, 87–91] and thus, although we have focussed on the phytoestrogenicity of individual compounds found in *Cyclopia*, we should consider the possibility that it is the mixture of compounds found in *Cyclopia* extracts, rather than an individual compound, that confers the desired estrogenic activity.

Acknowledgements

The authors would like to thank the Medical Research Council (MRC) and the Cancer Association of South Africa (CANSA) for financial support to A. L. (grant for projects entitled "*Cyclopia* Phytoestrogens" and "*Cyclopia* and breast cancer") and the Department of Science and Technology as well as the National Research Foundation (NRF) for financial support to E. J. (grant 70525). The views and opinions expressed are not those of the funding agencies but of the authors of the material produced or publicised.

Conflict of Interest

The authors declare no conflict of interest.

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Addendum B

List of publications and conference outputs.

B.1. List of publications

1. Robertson S, Allie-Reid F, Vanden Berghe W, **Visser K**, Binder A, et al. (2010) Abrogation of glucocorticoid receptor dimerization correlates with dissociated glucocorticoid behavior of compound A. J Biol Chem 285: 8061-8075.

2. **Visser K**, Smith C, Louw A. (2010) Interplay of the inflammatory and stress systems in a hepatic cell line: Interactions between glucocorticoid receptor agonists and interleukin-6. Endocrinology 151: 5279-5293.

3. Louw A, Joubert E, **Visser K**. (2013) Phytoestrogenic potential of *Cyclopia* extracts and polyphenols. Planta Med 79: 580-590.

4. **Visser K**, Mortimer M, Louw A (2013) *Cyclopia* extracts act as ERα antagonists and ERβ agonists, *in vitro* and *in vivo*. PLoS ONE 8(11): e79223. doi:10.1371/journal.pone.0079223

B.2. Conference outputs

1. **Visser K**, and Louw A. (2011) The molecular mechanism of action whereby phytoestrogenic extracts of *Cyclopia* modulates estrogen induction of breast cancer. *Poster presentation.* Spetses Summer School on Nuclear Receptor Signalling in Physiology and Disease, a FEBS (Federation of the Societies of Biochemistry and Molecular Biology) advanced lecture course cosponsored by CRESCENDO. Island of Spetses, Greece.

2. **Visser K**, and Louw A. (2012) The molecular mechanism of action whereby phytoestrogenic extracts of *Cyclopia* modulates estrogen induction of breast cancer. *Poster presentation.* Multidisciplinary conference supported by the South African Society for Biochemistry and Molecular Biology (SASBMB) and the Federation of African Societies of Biochemistry and Molecular Biology (FASBMB). Drakensberg, South Africa.